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Physiological Zoölogy

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ON THE ROLE OF SEX HORMONES IN SEX DIFFERENTIATION IN THE OPOSSUM (DIDELPHYS VIRGINIANA)[1]

(Six plates)

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I. INTRODUCTION

IT IS still a current problem to define, even within broad limits, the forces that are operative in sexual differentiation. Although it is generally agreed that sex determination occurs at fertilization, it is well known that the embryos of each sex, during development, exhibit definite features that characterize both sexes; at a given stage in development the gonads may be indistinguishable from each other, and both Wolffian ducts (male components) and Müllerian ducts (female components) are present and

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It is a great pleasure to acknowledge with a debt of gratitude the contribution of most valuable aid in this investigation by the following persons: to Mr. Clarence D. Cook, Lakeside, Michigan, for a supply of adult opossums, as well as females bearing pouch young; to Mr. C. B. Coursen, General Biological

equally developed, irrespective of the determined sex. Sexual differentiation subsequent to this "indifferent stage" may take a direct course toward maleness or femaleness or may take an intermediate course in the event of unusual environmental influences; we are now well aware of sex reversals induced at will in lower vertebrates by changes in the temperature of the developing medium (Witschi, 1939), by transplantations of heterologous gonads (Humphrey, 1929, 1935), and through parabiotic influences (Witschi, 1927; Burns, 1930, 1931). (For extended review of these phases see Willier, 1939, and Witschi, 1939.) In cattle twins (freemartin) evident humoral transmissions from males to females induce marked modifications in females, covering almost a complete range from female to male (Lillie, 1917; Willier, 1921; Bissonnette, 1924). It is plainly evident, therefore, that zygotic sex determination does not establish an irreversible predestination and that either sex, up to a certain period, is capable of some modification in the direction of the alternative sex. Under normal conditions some force, or set of forces, exercises a sex-differentiating role that gives clear expression to the dominant sex factors. But unsolved at the present time, are such problems as the period over which these forces operate, what limitations they have in producing ultimate effects, and what additional factors may play a role in sexual differentiation.

It is a general assumption that zygotic sex-determining factors act also as sex-differentiating factors, at least up to a stage when gonadal differentiation presumably assumes control. Gonads, therefore, would be dependent upon zygotic factors; but duct differentiation would depend upon substances secreted by the differentiating gonad rather than upon the same forces that operate to produce the distinctive gonad. In this respect an adjunct to sexual differentiation would appear in vertebrates that is not present in invertebrates, just as sex hormones from the gonads in the mature stage control reproductive systems in vertebrates and not in invertebrates.

One of the first and clearest cases indicating that hormones play the role of duct differentiators was the case of the freemartin described by Lillie (1917). The incontrovertible facts in the case were ingeniously interpreted by Lillie on the basis of the then accepted contentions with regard to hormone action, some portions of which require modification on the basis of more recently established facts.

Subsequent to the preparation of pure chemical substances that act in adults as hormones, some of which may be the true gonad hormone that controls the adult reproductive system, various attempts have been made to determine their effects upon embryonic sex differentiation and to put to a test the theory that sexual differentiation is dependent upon such substances (Dantchakoff; Kozelka and Gallagher; Willier, Gallagher, and Koch; Wolfe and Ginglinger; Raynaud; Greene, Burrill, and Ivy; Turner; Burns; Moore). It is to be admitted that, even if the conception is true that hormones secreted by the developing gonads are responsible for duct differentiation, there is still no assurance that the substances exercising a morphogenetic role in the embryo are the same as those operating on the functional system of the adult.

Supply Company, for females bearing pouch young and for adults, supplied at cost; to Dr. Dorothy Price for invaluable aid in treatments and in handling materials; to Miss Sally Johnson and Mrs. Suzanne Claussen for excellent microscopical preparations; to Drs. G. Stragnell and E. Schwenk, Schering Corporation, for testosterone ointment, *l*-propionate ointment, and oil solutions of progynon-B; to Dr. George Cartland, Upjohn Company, for gonadogen; to Dr. Donald Wonder, Cutter Laboratories, for gonadin; to Dr. Oliver Kamm, Parke Davis and Company, for theelin; to Mr. Kenji Toda for preparation of drawings and photomicrographs; and to Dr. Joseph Schwab for photographs of live young in the marsupial pouch (Figs. 1 and 2).

The writer's interest in this problem was aroused by a simple suggestion in 1917 by Professor Frank R. Lillie to consider making an attempt to produce a freemartin effect by experiment. Such efforts, begun at once, have led into many bypaths in the course of their developments; but the main problem has been reapproached repeatedly, the principal object being to subject the differentiating reproductive system to hormones of the opposite sex. Such attempts on my part have involved, in order, (1) testis transplantation into pregnant females, (2) parabiosis between normal males and pregnant females, (3) transplantation of gonads into embryos *in utero*, (4) transplantations of developing embryonic tissue into postnatal hosts of different hormonal states, (5) injections of testis extracts in oil into the amniotic cavity, and (6) injections of chemical androgens into pregnant mothers. Experiences with the latter project, which was begun in 1937 and which involved injection of testosterone propionate into pregnant rats (Moore, 1940), suggested the desirability of some other experimental animal. It developed very early that injections of *l*-propionate into pregnant female rats led to the death of a large proportion of embryos by the 20th day of gestation, the birth of dead or weakened embryos, delayed parturition, and faulty lactation. The fact that injections of pregnant mothers led to the death of some or all embryos in 60 per cent of the cases caused some concern as to whether any noted departures from the normal could be attributed to specific hormone action in the embryo or whether it might be due to abnormal development as a direct result of nonspecific placental injury. Confronted with this problem, an effort was made to obtain experimental materials not subject to the same unknown possibilities; and in February, 1938, opossum pouch young were obtained to study the suitability of this material. A preliminary account of the effects of sex hormones on sexual differentiation has been published (Moore, 1939), as well as a short account dealing with the care of captive animals, their breeding periods, and growth of young (Moore and Bodian, 1940). Entirely independently, and almost simultaneously, Burns (1939*b*, 1939*c*) published his preliminary findings from similar treatments on pouch-young opossums and has since published more detailed accounts of his experiments (1939*d*, 1939*e*).

The advantages of marsupials for such studies will be obvious at once. The opossum young at birth is sexually undifferentiated, possesses functional Wolffian ducts for approximately 2 weeks, shows beginning development of Müllerian ducts on the 3d day, and their contact with the urogenital sinus by the 10th day; the gonad becomes recognizable as testis or ovary on or about day 3. The young, firmly attached to nipples in the marsupial pouch, remain readily accessible for direct treatment rather than indirect treatment through the mother. Although they are postnatal young, their precocious development, in some respects, makes them available for direct treatment at a period in sexual differentiation that corresponds with fetal development in placental mammals.

It has been anticipated that a study of the effects of hormones applied to developing embryos may provide a logical basis for a re-examination of the thesis that sexual differentiation, especially that of the duct system, rests upon a directional humoral influence (hormone or hormones) provided by the developing gonad; however, one must not lose sight of the differentiating mechanism responsible for directing development of the gonad itself. Whether such an influence, zygotic in contrast to humoral, fails to operate on duct differentiation and whether such forces are supplanted by a newly produced humoral substance of gonadal origin are the essential questions of the main problem under investigation.

II. THE MATERIALS

In another place some experiences have been related in obtaining pouch-young opossums for experimental work (Moore and Bodian, 1940). Briefly, for present purposes, adult opossums confined in outdoor pens on the grounds of the Whitman Laboratory on the campus have delivered 20 litters whose birth was known within 24 hours, and from these litters 124 young were obtained (see Pl. I, Figs. 1 and 2). Some of these young were removed from the pouch periodically, carefully measured as to neck-rump length, head length, and weight. They provided a key series with which young of unknown delivery could be compared and their ages estimated with a high degree of accuracy. Seventeen females bearing pouch young were received through shipments by express from the states of Louisiana, Florida, Arkansas, Missouri, Illinois, and Michigan, the young in most cases less than 15 days of age. These females, likewise, provided 124 pouch young whose ages were estimated by comparison with known deliveries. From these 248 pouch young a normal series of 65 stages has been preserved in Bouin's fluid, and the reproductive tract prepared in serial section. The age range from day 1 to day 100 includes more than one-half of the stages during the first 30 days of development in the pouch. Of the 65 stages, constituting the normal untreated series, 21 are of known age, 44 of estimated age. Some stages are represented by a single animal; several stages by both a male and female, in many instances littermates; while some of the stages are represented by 3–5 different individuals. One definite age is occasionally represented by young from 5 different females.

In the hormone-treated series, no account is taken of the many young whose deaths occurred during treatment; estrogens, especially, have proved decidedly toxic, and records and preservations here considered account only for those contributing to the problem. Subcutaneous injections are fairly satisfactorily done subsequent to day 15; but, owing to the small size of the young (25–30 mm.), quantitative accuracy cannot be attained; some seepage occurs frequently from the injection site. Prior to day 15 hormones have been administered largely as a cream ointment applied over the entire surface of the body by means of a small brush; the hormone is readily absorbed through the skin (see Moore, Lamar, and Beck, 1938). It follows that the entire number of young in each pouch must be treated alike to prevent contamination by contact between embryos treated differently or between untreated ones in contact with treated.

Androgens.—A lanolin cream ointment carrying free testosterone or *t*-propionate, as well as testosterone propionate in sesame oil, has been employed. Animals preserved following treatment with androgens represent variable lengths of treatment as well as treatments during different age periods. A total of 35 androgen-treated young, preserved at ages from day 16 to day 83, consists of 16 young from known-age litters and 19 of estimated age; 15 males and 20 females receiving treatment have constituted the bulk of materials from which effects are depicted; but other single young, fairly accurately estimated as 8-month and 1-year specimens, have afforded supplementary material.

Estrogens.—Estradiol treatment by ointment has been the principal means by which estrogenic substances were applied, but in addition theelin in oil and Progynon-B have been injected subcutaneously. Several entire litters have been lost during treatment, because of toxic effects of estrogenic substances. Young are frequently found detached from the nipple and dead in the pouch or cage at the rate of 1–3 daily, whereas, the day previous all young appeared healthy. Decreasing the dosage has resulted in obtaining

several young that have withstood treatment for sufficient periods to be instructive. Preserved young treated with estrogens consist of 25 specimens, of which 21 were of known age, 4 of estimated age. Preservation was done between ages of 12 and 88 days, and the group contains 10 males and 15 females.

Gonadotropic substance.—Gonadogen and gonadin, each representing the gonadotropic activity from the serum of pregnant mares, were used in the case of a few small litters, but the materials in this treatment have been less adequate than with sex hormones. Seven young were preserved between the ages of 17 and 38 days, all being from known-aged litters and consisting of 3 males and 4 females; in addition to these 1 male and 1 female of an estimated age of 100 days received treatments.

III. NORMAL PROGRESSION IN SEX-DUCT DIFFERENTIATION

INTRODUCTION

The adult reproductive systems of the opossum, male and female, are shown in Figures 3 and 4 (Pls. II and III). The special features of the male to which attention should be directed are the absence of seminal vesicles and the presence of the inclosed type of prostate gland; rather than a discrete, well-circumscribed prostate gland, this type consists of numerous branched, tortuous glands opening into the urogenital canal and confined within the sheath surrounding this structure. This gland in the opossum has been studied recently by Chase (1939), to whose work the reader is referred. The female system is peculiar because of the fact that the paired Müllerian ducts never fuse posteriorly to form the body of the uterus, but, rather, each Müllerian duct remains separate; it should be emphasized that, since Müllerian ducts fail to fuse in either sex, males never exhibit a uterus masculinus. The lower ends of the Müllerian ducts, posterior to the point of approximation and usual fusion in placental mammals in the genital cord, undergo a special development (along with horns of the urogenital sinus) to constitute the lateral vaginal canals—a peculiarity of the marsupial reproductive system. Whereas spermatozoa ascending through the urogenital canal ("middle" or "posterior vagina" [Hartman, 1923]) pass through the lateral vaginal canals into the uterus proper and on to the oviducts, at parturition marsupials develop a new birth canal by means of a rupture of the uterus at the cervix; a passage is formed through connective tissue that enters into the urogenital sinus, and the fetus thus does not pass through the lateral vaginal canals. This peculiar birth procedure was first described by Hill (1900) for *Dasyurus*.

To determine the effects of hormones upon sexual differentiation, it became necessary to establish the normal sequence in development for both males and females. The writer is unaware of a satisfactory account of the details of male development in the opossum, though much information has been added by the study of Chase (1939). For female differentiation the recent account of Baxter (1935), while highly satisfactory in most respects for Müllerian duct development, leaves out of account the Wolffian system or its derivatives, and for the purposes of this investigation these are very important. Furthermore, it is highly desirable to have at hand normal stages in the development of both sexes seriated as to age in the same manner as experimental embryos are seriated. This assures a knowledge of the condition of structures present at the time of beginning treatment. McCrady (1938) deals primarily with prenatal development, but a more recent study by McCrady (1940) treats the later development of the urogenital sinus and phallus. Since it is expected that a more detailed description of portions of normal differentiation will form the subject matter of other papers, expecially the development of go-

nads, only sufficient detail on normal stages will be sketchily presented here to provide a background of development for comparison with treated stages.

Preservation of normal young must be at random as to sex until about day 12, when a careful examination of the young attached to nipples in the pouch permits a selective removal of sexes. Males at this time begin to show a slight scrotal elevation anterior to the phallus (the male opossum possesses a scrotum located well anterior to the penis), and females exhibit a definite mammary-gland pattern in the developing pouch that is clearly diagnostic of sex; rudimentary nipple anlagen are less prominent on males. Covering the first 16 days of pouch development, the normal series upon which this account is based contains 40 pouch young, 17 of known date of birth and 23 of estimated age; the group includes 15 males and 14 females, and the remainder were undetermined as to sex. Subsequent to day 16, many stages are represented by littermate males and females (days 24, 32, 44, 67, 75, 84, and 100). Intermediate stages are represented both by isolated males and females, and in some cases the same age is represented by several young of the same sex. Often large litters yielded two or three successive stages for the normal series before the remainder were assigned for treatments with hormones. Where possible, males and females of the same litter were selectively reserved for treatment.

The opossum young at birth (see Pl. I, Fig. 1) is sexually undifferentiated, but by the 5th day in the pouch gonads are assuming definite testicular or ovarian characteristics. Both sexes possess well formed and functional Wolffian ducts, since a functional mesonephros is present until approximately day 15; subsequent to this period, progressive mesonephric degeneration occurs, and the metanephros gradually becomes the functional excretory organ.

Müllerian ducts are discernible in the 3-day pouch young. A fairly distinct ostium is continued, usually by 3–6 sections of an epithelial cord with barely visible lumen, and further continues posteriorly as a solid cord of cells approximately one-half the length of the mesonephros, where it is lost. Day 10 introduces the beginning of sexual dimorphism in duct development. Because of its urinary function, the mesonephric duct is similar in both sexes, but Müllerian ducts exhibit slight sex differences.

10TH DAY

A. *Males.*—The male opossum pouch young of 10 days postnatal development exhibits a testis with definite seminiferous cords, a developing rete cavity with cords passing toward the mesonephros, a fully functional mesonephric duct that enters the urogenital sinus, and Müllerian ducts. The diameter of the lumen of Wolffian ducts in serial sections varies in its course from 0.04 mm. to 0.06 mm.

The Müllerian system is visible in the 10-day male as a fairly distinct ostium, from which an epithelial cord with only an occasional tiny lumen runs through the duct portion of the mesonephros. Posterior to the mesonephros, the Müllerian cord passes more indistinctly toward the genital cord but cannot be followed to contact with the urogenital sinus epithelium.

B. *Females.*—Females at 10-day pouch development differ from males (aside from the recognizable ovary) by the fact that the Müllerian system of ducts exhibits somewhat better development. The ostium is more definite; a very small lumen (0.009 mm.) is present throughout the upper two-thirds of the mesonephros. Posterior to this region the nonluminate epithelial cord leaves the mesonephros, passes to the genital cord alongside the

Wolffian duct, and its cells can be traced to contact with the urogenital sinus epithelium at the point of entry of Wolffian ducts.

DAYS 15 AND 16

The 15th or 16th day of pouch development is a fairly important age for the introduction of a few cardinal changes. At this time the metanephros exhibits well-formed tubules and the organization of the glomeruli has progressed remarkably. The mesonephric glomeruli, especially at the anterior end, exhibit marked disorganization. The entire renal corpuscle is often hyaline in nature, and the tubules exhibit marked shrinkage and fragmentation. By this period the metanephric duct (ureter) is larger and exhibits a more definite entrance into the urogenital sinus than does the Wolffian duct. In short, the metanephros is gaining in ascendancy as the mesonephros declines, and it is probable that at this period both are active in excretory function.

A. *Males.*—Representing this stage of development are 2 males of day 14, 3 of day 15, and 4 of day 16. The testicle exhibits definite and well-formed seminiferous cords; the rete presents a distinguishable, though small, ramifying rete cavity; and nonluminate cords of cells from the mesonephros make contact with it.

1. Wolffian ducts: In each of the 9 males of these 3 days of development the Wolffian duct retains a well-formed lumen within the mesonephros (luminal diameters, 0.06–0.03 mm.) As the duct enters the genital cord, it becomes progressively reduced, and in all 9 pouch young the entrance into the cavity is approaching extinction; a definite entrance is still present, and the diameter of the lumen in the genital cord is reduced to 0.007–0.01 mm.

2. Mullerian ducts: The ostium, at this time, is either extinct or unrecognizable. In each of the 9 males the ducts are represented by solid cords of cells in the mesonephros; and in the majority the cord is visible posteriorly, just ventral to the Wolffian duct, passing in the direction of, but unrecognizable in the substance of, the genital cord. A lumen is scarcely recognizable at any point along the course of the cord in any of the 9 males.

3. Prostate: This stage of development marks the first appearance of the prostate anlage in male embryos; in female opossums a prostate anlage is never recognizable at this, or any stage; hence, the structure is not represented in normal females—a point of especial importance in this investigation. The two 14-day-old males do not show recognizable prostate proliferations from the urogenital sinus epithelium. Of the three 15-day males, two show definite beginnings of local bud proliferation representing the structure, whereas the third does not show it. Each of the four 16-day males shows a very definite anlage for the prostate, which appears as definite knobs or buds from the basal layer of the urogenital sinus epithelium (see Fig. 10 for 32-day male).

B. *Females.*—This period is represented by one 14-day female, two 16-day females, and a single 17-day female.

1. Wolffian ducts: The Wolffian ducts of the 14-day female and one of the 16-day females were similar to those present in the 9 males of equivalent age, in that a well-preserved duct still exists in the mesonephros with a lumen diameter of 0.04–0.06 mm., and this tapers in the genital cord to a barely perceptible entrance of the lumen into the urogenital sinus. The second 16-day female was similar, with the exception that the lumen of the duct was interrupted in the genital cord and failed to enter the urogenital sinus. In the 17-day female not only was the Wolffian duct lumen interrupted at the urogenital sinus but the cord stopped short of these structures.

2. Müllerian ducts: In females of this period the Müllerian ducts exhibit definite but very small lumina to the level of contact with urogenital sinus epithelium. At no stage in normal female development up to the 100th day is the lumen of Müllerian ducts traceable into the cavity of the urogenital sinus. Baxter (1935) has clearly demonstrated that the distal portion of the adult female tubes owes their origin to the horn of the urogenital sinus; and during this organization of the duct, connections with the urogenital sinus are represented by a solid epithelial mass.

C. *Résumé.*—These rather brief notations emphasize that Wolffian ducts at the 15- and 16-day stage of development are beginning to lose their luminal connections with the urogenital sinus in both sexes—an indication that their former definite connections are associated with excretory function and that no perceptible difference is seen between males and females. They likewise call to mind that the Müllerian ducts in males, as solid cords, are inferior to females and now fall short of the genital cords, whereas in females they are luminate and make contact with urogenital sinus epithelium. Further sexual differences are a definite prostate anlage in males and an entire absence of this structure in females.

DAY 24 IN THE POUCH

For the purpose of emphasizing the beginning sexual dimorphism indicated as of day 15, attention should be directed to this progress a few days later, and for that purpose attention will be called to a littermate male and female removed from the pouch on day 24.

A. *The male.*—The testis is now better organized, with well-developed solid seminiferous cords. The rete is represented by a heavy mass of epithelial cells penetrating among the seminiferous cords in the testis and making contact with these. At the opposite end mesonephric tubules, some with tiny cavities representing lumina, are traced to contact with the rete. The rete cavity is definite though not of great extent.

1. Wolffian ducts: At this stage the Wolffian ducts are appreciably reduced in size in the substance of the mesonephros and retain lumina with the greatest diameter 0.03 mm., or approximately one-half that of the 15-day stage. The mesonephros is now almost totally devoid of Malpighian corpuscles, and mesonephric tubules are, for the most part, absent or atrophic. Posterior to the remnant of the mesonephros the small cells of the Wolffian duct are reduced to a solid cord for some distance anterior to the urogenital sinus. This active regression of the Wolffian duct in the normal male, subsequent to the loss of function as a urinary duct, suggests that its differentiation or maintenance up to this period is not conditioned by hormone secreted by the testis.

2. Müllerian ducts: These are small nonluminate cords in the region of the degenerating mesonephros and no longer extend into the genital cord. The atrophy of these ducts in normal males is from the posterior toward the anterior end.

The prostate gland is much advanced in its development since the 15-day stage. It is represented by numerous large buds and epithelial knobs growing out from the urogenital sinus epithelium (see Pl. V, Fig. 10, for prostate in 32-day male).

B. *The female.*—

1. Wolffian duct: In normal 24-day females the Wolffian duct is reduced to a solid cord of cells throughout most of its extent, but in a few sections located at the gonad level a very small lumen is present. This small cord of cells is lost anterior to the urogenital sinus.

2. Müllerian duct: The Müllerian duct of females is a well-formed tube composed of a relatively thick epithelium and contains a lumen through most of its course. At the anterior end the lumen is very small, but more posteriorly it shows a slight enlargement indicative of the future uterine portion. In the genital cord the two tubes approach closely in the mid-ventral line at the region known as the cervix (Pl. III, Fig. 4); fusion, however, does not occur in the opossum female at any stage of development. Posterior to the cervix region, the tubes become decidedly more separated and begin to show a slight, though definite, enlargement for the lateral vaginal canals. The lumen of this portion is further traceable toward contact with the urogenital sinus; but, as is usual, this portion is represented by a solid mass of cells continuous with the sinus horn epithelium.

Thus, by the 24-day stage the female tract is marked off into its future parts of oviduct, uterus, cervix, and lateral vaginal canals. Figure 5 (Pl. IV) reveals the dissected female system at 31 days; and Figure 7 (Pl. V) reveals sections of the cervix and uterine level of the Müllerian duct.

Cowper's gland, which arises in both sexes at approximately day 9 as outgrowths from the distal portion of the urogenital sinus epithelium, appears somewhat further developed in the male of the 24-day stage than in the female. In each case the anlage is still a solid cord of cells extending away from its point of origin and beginning to show branchings.

44TH DAY

A. *The male.*—The testes of 44-day males are quite characteristic of inactive testes whose seminiferous cords are distinct and contain layers of cells shifting peripherally and suggesting a stage prior to the development of a lumen; no lumen is yet evident, however, and none will appear until the animal approaches the breeding season of the year following birth.

The epididymis in section consists of well-defined cords, some of which exhibit a definite lumen. The tubules can be traced to contact with the rete testis, which now shows a well-defined rete cavity at the position of the future hilus of the organ. It appears that a single tubule from the epididymis makes contact with the rete cavity, but this becomes subdivided into several epididymal tubules outside the testis.

The vas deferens is traceable as a tube from the epididymis to within a few sections anterior to its contact with the epithelium of the urogenital sinus; no luminal connection exists between the duct and the sinus cavity. Diameters of the lumen range from 0.01 to 0.03 mm.

Müllerian ducts are reduced to solid cords of epithelial cells from a point immediately below the epididymis to approximately one-half the length of the vas deferens, beyond which it is no longer recognizable.

The prostate gland is represented by well-circumscribed solid cords of cells growing out from all sides of the urogenital sinus epithelium, some showing definite branching.

B. *The female.*—The ovary at 44 days has advanced in development and is quite different, in detail, from the 24-day stage. The epoöphoron has exhibited marked development; and, whereas it is somewhat smaller as regards number of cross-sections of tubules, these are approximately in the same grade of development as those of the male; cords are traceable through the mesovarium to the somewhat enlarged rete cavity located within the developing medulla, surrounded by a cortex that exhibits decided activity.

The Wolffian ducts of the female are reduced to nonluminate cords of poorly organized

cells, which continue, along with the Müllerian ducts, approximately two-thirds the distance to the genital cord.

Müllerian ducts have progressed to the point of very definite differentiation of parts—oviducts, uterus, cervix, and lateral vaginal canals. Oviducts have become tortuous in their course and show a greatest luminal diameter of 0.03 mm. The uterine portion is decidedly expanded and exhibits a luminal diameter up to 0.30 mm. The epithelium is yet simple, and no glandular outgrowths are evident. The constricted cervical portion of the tube is narrowed to a diameter of 0.07 mm. and continues into the lateral vaginal canals, which are also expanded until their luminal diameters reach 0.37 mm. Posteriorly the tubes continue to contact with the urogenital sinus epithelium, the posterior portion being composed of a solid epithelial cord mass.

Cowper's gland exhibits marked differences in the two sexes at this stage. In the female this structure consists of well-formed heavy solid cords growing out from the urogenital sinus epithelium. In the male the outgrowth is approximately twice as large, shows more marked branchings, and is luminate.

DEVELOPMENT UP TO 100 DAYS

It will suffice, for the present account, to outline in a brief manner the further development of the normal reproductive system up to the 100th day, which will largely cover the ages subjected to artificial hormonal influences.

A. *Males.*—Testis differentiation continues slowly up to the 100th day, at which time the seminiferous tubules consist essentially of a single basal layer of cells representing the germinal epithelium but no development of tubular lumina. In 8-month-old males seminiferous tubules are luminate, and the germinal epithelium is actively producing sperm heads and spermatozoa; 1-year animals show greater quantities of spermatozoa.

The vas deferens between the 44th and 100th day increases gradually in size and in the differentiation of its walls; the greatest luminal diameter of 0.03 mm. at day 44 reaches 0.04 mm. by day 67, 0.06 by days 83–100. The urogenital sinus end is consistently closed by a solid epithelial mass representing the urogenital-sinus-horn portion of the duct; at day 100 the lumen is traceable into the cavity of the urogenital sinus.

Prostate-gland development involves a gradual increase in size of the entire mass of the urogenital sinus, with the prostatic outgrowths becoming more profusely branched: these outgrowths from the sinus epithelium begin to exhibit slight cavities at their medial ends by day 83. Growth indications were recorded as diameters of the structure between the developing muscle layers, rather than total outside diameter, in order to portray, as nearly as possible, prostatic growth. These diameters have increased from 0.5 mm. on day 40 to 1.0 mm. on day 75, 1.5 mm. on day 100, 2.7 mm. at 8 months, and 9.0 mm. at 1 year. The external character of the adult prostate will be seen from Figure 3 (Pl. II), and a section of a 100-day prostate in Figure 14 (Pl. V). A section of the prostate of the 32-day stage is shown in Figure 10 (Pl. V).

Müllerian ducts in males, which at day 44 were represented by a solid cord of cells extending approximately one-half the distance from the epididymis to the urogenital sinus, show increased growth activities in the remnants subsequent to this period. On day 67 the anterior portion of this cord displays a lumen having a diameter of 0.01 mm. This continues its development until, on day 83, the entire remaining portion of the persisting cord contained a lumen of 0.01–0.03 mm. A similar condition holds on day 100

with a diameter of 0.04, and in one specimen a dilated portion measured 0.11 mm. Each end of the tube located along the developing epididymis was closed.

B. *Female.*—Ovarian development involves many changes in its final differentiation, the subject of a future communication; but by day 100 well-developed follicles containing large ova are present; no antrum-bearing follicles appear even at the 8-month stage of development. Peculiar syncytial masses, formerly making up the principal portion of the cortex area and designating an incomplete development, are still evident at day 100.

The epoöphoron continues its development subsequent to day 44 and reaches relatively large proportions in the opossum female. Connections exist between the cords or tubules of the epoöphoron and the rete ovarii, and the latter exhibits a relatively large cavity within the ovarian medulla of the opossum ovary. Epoöphoron tubules also connect with the Wolffian duct, which remains at the 100th day in the upper portion of its course; and, whereas the duct is devoid of a lumen on day 44, growth and further differentiation involve the gradual reformation of a lumen in the 100-day female of 0.09 mm. in diameter.

The Müllerian system continues its development from day 44 by increases in the folds of the oviducts, by uterine enlargement, and by enlargement of the lateral vaginal canals. Oviducal lumina increase from 0.03 mm. on day 44 to 0.11 mm. on day 100.

Uterine development by day 100 (Pl. VI, Fig. 22) involves gradual increases in luminal diameters from 0.30 mm. on day 44 to 1.05 mm. Glandular outgrowths later make their appearance as proliferations of the lining epithelium; and by the 8-month stage the uterus exhibits a much vacuolated, cheesy type of development due to the tortuosity of the glands.

Lateral vaginal canals have similarly increased perceptibly in size and differentiation and are demarcated from the lower uterus by the narrow cervical portion. The lumen of the lateral vaginal canal attains a diameter of 0.90 mm. by day 100, whereas that of the cervix is 0.07 mm.

Cowper's gland in 100-day males and in females has continued its differentiation as a result of further development of epithelial buds, their branching, and the appearance of cavities within the local portions.

IV. EFFECTS OF ANDROGENIC TREATMENTS

Androgens have been administered daily to male and female pouch young as an ointment of testosterone and of *l*-propionate and by subcutaneous injection of *l*-propionate in sesame oil. Treatments prior to day 15 have usually been by ointment, owing principally to the difficulty of injecting very small animals; in a few cases hormones in alcoholic solution were applied on the skin, but this treatment was discontinued because of the greater loss of young from the pouch. Some young have been injected subcutaneously by the 10th day, when the total body length was 20–25 mm. and the total weight 0.8 gm., but injections subsequent to day 16 were more satisfactory; as the animals grew, injections naturally became progressively simpler. Dosages by inunction have been variable and are difficult to approximate with any precision, since greater quantities of ointment can be applied as the animal increases in size. Subcutaneous injections prior to day 70 have been given in total daily doses of 0.25 mg. of *l*-propionate in 0.05 cc. of sesame oil, but unavoidable seepage from the skin puncture occurred frequently. Subsequent to day 70, total daily doses have been 0.5 mg. in some cases and 5.0 mg. in others.

Without consideration of animals lost during treatment, 18 males have been preserved

at ages from 16 to 100 days, and several additional ones were treated at ages of 8 months and 1 year. Twenty-two androgen-treated females were preserved at the close of treatment. From this group of 40 preserved, androgen-treated animals, in addition to still older ones, 13 males and 16 females have been selected for brief tabulation in Table 1. This table shows types of treatment, age at preservation, and responses represented by ocular micrometer measurements of structures from serial sections, as well as subjective indications of relative response of structures. Selection for tabulation was made to include animals treated by the three methods of hormone administration, and in particular to include littermate males and females. Males and females in the table designated by the same number (i.e., ♂ 96A, ♀ 96B) are in every case littermates.

A. *Effects on males.*—The most outstanding effect of androgenic treatment on pouch young has been the stimulation of the external genitalia and of the prostate gland and of Cowper's gland; since the latter structure will be considered in more detail in another place, it will receive only brief mention here. Slight stimulation only has been seen on Wolffian-duct and Müllerian-duct development in males. No inhibitory effects have been noted in any case. Attention is here called to Table 1 for a brief notation of responses.

1. Wolffian duct: Responses of the Wolffian duct in androgen-treated males have been surprisingly minimal. It should be recalled that in normal males definite but partial Wolffian duct involution follows loss of the urinary function of the mesonephros. The diameter of the lumen of this duct decreases from a maximal of 0.09 mm. on the 12th–14th days to approximately 0.04 mm. from days 16–18 up to about day 83. The lumen of the duct can be traced into the urogenital sinus up to approximately day 16; but, subsequent to this period, the duct entrance is closed by a solid epithelial cell mass, associated apparently with modifications of the urogenital sinus horns with which they connect (see Baxter, 1935). Indications given in this table suggest that in 3 of the 12 treated males there was a slight stimulation of the Wolffian duct; judgment of such stimulation was based largely on the condition of the duct and the size of the lumen in treated males compared with normal males of similar age. Such larger luminal diameters are frequently fairly localized and fail to extend over the entire course of the duct. It is to be emphasized that in no case did treatment with androgenic substances prevent the reduction in size of the duct subsequent to the loss of its urinary function, even though treatment was begun many days prior to mesonephric degeneration. A section of the Wolffian duct (vas deferens) of the 31-day male 64A, treated with *t*-propionate by subcutaneous injections from day 10 to day 30, can be seen in Figure 9 (Pl. V): it does not reveal stimulation, in contrast to a much stimulated prostate (compare prostate with normal ♂, Pl. V, Fig. 10) and the greatly stimulated external genitalia. In a similar manner androgenic treatment failed to maintain luminal connection of the duct with the cavity of the urogenital sinus. At the age of 83–100 days, treatment appears to hasten the re-establishment of luminal connections with the sinus, but the evidence is considered none too definite.

In contrast to the duct (vas deferens), the epididymal tubules (mesonephric) appear to respond to androgens, exhibiting more tortuous tubules, better lumina, and better tubule walls. No comparative measure has been found entirely satisfactory to express these differences; hence, they must be based upon subjective judgment.

2. Müllerian duct: The Müllerian duct in males responded more definitely to androgenic treatment than did Wolffian ducts, but this may be associated with a much greater involution of the former. To recall the condition in normal males, the Müllerian duct

TABLE 1

EFFECTS OF ANDROGENS ON OPOSSUM YOUNG*

SER. NO.	AGE AT DEATH (DAYS)	TREATMENT (DAYS)	WOLFFIAN DUCT	WOLFFIAN-DUCT STIMULATION	PROSTATE UROGENITAL SINUS	PROSTATE STIMULATION	MÜLLERIAN DUCT					PHALLUS
							Oviduct	Uterus	Cervix	Lateral Vaginal Canal	Stimulation	
	Males											
96A	16	3–15 TO	0.03 (0.06)	o	0.60 (0.60)	+	.....	0.00 (0.00)	.....	.....	o	2.2×1.2
63A	22	10–21 TPI	0.04 (0.03)	o	0.90 (0.63)	+	.....	0.01 (0.00)	.....	.....	?	2.0×1.8
47A	24	3–24 TO	0.02 (0.03)	o	1.05 (0.63)	+	.....	0.00 (0.00)	.....	.....	o	3.0×2.3
38B	25	15–25 TPO	0.03 (0.03)	o	0.90 (0.63)	.....	.....	0.00 (0.00)	.....	.....	o	2.1×1.5
27A	27	16–27 TO	0.07 (0.03)	+	0.75 (0.63)	+	.....	0.00 (0.00)	.....	.....	o	.....
101A	30	3–15 TO 16–29 TPI	0.09 (0.03)	+	1.42 (1.05)	++	.....	0.01 (0.00)	.....	.....	+	3.0×2.1
64A	31	10–30 TPI	0.03 (0.03)	o	1.27 (1.05)	++	.....	0.03 (0.00)	.....	.....	+	3.1×2.5
31A	32	16–31 TO	0.04 (0.03)	o	0.90 (1.05)	+	.....	0.01 (0.00)	.....	.....	?	3.0×2.0
30A	32	16–31 TPI	0.11 (0.03)	+	1.12 (1.05)	++	.....	0.04 (0.00)	.....	.....	+	3.0×2.2
43B	32	16–31 TPO	0.03 (0.03)	o	1.12 (1.05)	+	.....	0.00 (0.00)	.....	.....	o	2.8×2.0
65A	39	10–31 TPI 32–39 no tr.	0.06 (0.03)	o	1.42 (1.05)	+++	.....	0.09 (0.00)	.....	.....	+	3.7×3.0
92	83	70–82 TPI	0.04 (0.06)	o	2.85 (1.35)	+++	.....	0.03 (0.03)	.....	.....	o	7.5×3.5
98	100	70–99 TPI	0.07 (0.07)	o	4.9 (1.50)	++++	.....	0.03 (0.05)	.....	.....	o	15.5×6.0

* Phallic measurements from 80 per cent alcohol before sectioning. Prostatic measurements, greatest diameter of cross-section of urogenital sinus and prostate outgrowths. Other measurements, maximal diameter of duct lumina (ocular micrometer measurements from sections). All measurements expressed in millimeters. Figures in parentheses represent normal embryos. Stimulation: +, slight; ++, moderate; +++, great; ++++, extreme; o, no effect; no tr., no treatment; TO, testosterone ointment; TPO, *t*-propionate ointment; TPI, *t*-propionate injection.

TABLE 1—*Continued*

Ser. No.	Age at Death (Days)	Treatment (Days)	Wolffian Duct	Wolffian-Duct Stimulation	Prostate Urogenital Sinus	Prostate Stimulation	Müllerian Duct					Phallus
							Oviduct	Uterus	Cervix	Lateral Vaginal Canal	Stimulation	
	Females											
96B....	16	3–15 TO	0.03 (0.06)	o	0.48 (0.60)	+	.....	0.03 (0.00)	.....	.....	+	2.2×1.2
63B....	22	10–21 TPI	0.04 (0.00)	+	1.12 (0.75)	++	0.03 (0.00)	0.49 (0.01)	0.07	1.05 (0.01)	+++	1.8×1.4
47B....	24	3–24 TO	0.06 (0.00)	++	0.90 (0.75)	+++	0.03 (0.00)	0.11 (0.01)	0.03	0.30 (0.01)	++	2.2×1.8
38A....	25	15–25 TPO	0.00 (0.00)	o	0.90 (0.75)	+	0.03 (0.00)	0.06 (0.01)	0.03	0.12 (0.01)	+	1.7×1.2
27B....	27	16–27 TO	0.06 (0.00)	+	0.75 (0.75)	++	0.01 (0.00)	0.09 (0.01)	0.02	0.07 (0.01)	+	.....
101B....	30	3–15 TO 16–29 TPI	0.11 (0.00)	++	1.20 (1.05)	+++	0.07 (0.03)	0.65 (0.07)	0.06	1.42 (0.11)	+++	4.0×2.5
64B....	31	10–30 TPI	0.26 (0.00)	+	1.42 (1.05)	+++	0.03 (0.03)	1.35 (0.07)	0.03	2.70 (0.11)	++++	3.0×2.5
31B....	32	16–31 TO	0.04 (0.00)	+	0.90 (1.05)	++	0.03 (0.03)	0.18 (0.07)	0.03	0.26 (0.11)	++	2.4×1.8
30B....	32	16–31 TPI	0.05 (0.00)	+	1.27 (1.05)	+++	0.07 (0.03)	0.30 (0.07)	0.03	1.42 (0.11)	+++	3.1×2.1
43A....	32	16–31 TPO	0.03 (0.00)	+	1.12 (1.05)	++	0.03 (0.03)	0.33 (0.07)	0.03	0.57 (0.11)	++	2.0×1.8
65B....	39	10–31 TPI 32–39 no tr.	0.37 (0.00)	+	1.50 (1.20)	++++	0.05 (0.03)	1.50 (0.30)	0.02	1.60 (0.37)	+++	3.0×2.7
107.....	45	3–15 TO 16–44 TPI	0.07 (0.00)	++	1.95 (1.20)	+++	0.04 (0.03)	0.22 (0.30)	0.03	0.52 (0.37)	+++	4.6×3.0
66.....	62	10–31 TPI 32–62 no tr.	0.75 (0.00)	++	2.25 (1.20)	++++	0.07 (0.03)	1.65 (0.60)	0.04	0.60 (0.90)	+++	3.0×3.2
117.....	95	3–15 TO 16–93 TPI	0.02 (0.00)	+	5.50 (1.35)	++++	0.03 (0.03)	0.82 (1.05)	0.07	0.90 (0.90)	++	7.8×5.5
118.....	95	3–44 TPI 45–95 no tr.	0.37 (0.00)	++	2.10 (1.35)	++++	0.04 (0.04)	0.30 (1.05)	0.07	1.50 (0.90)	+++	5.2×1.8
120.....	147	3–95 TPI 96–147 no tr.	0.70 (0.00)	++	3.60 (....)	++++	0.05 (....)	0.82 (....)	0.07	0.90 (....)	+++	9.6×5.5

appears on day 3, gradually extends posteriorly as a solid cord of cells which reaches the genital cord on days 8–10; but the duct fails to make contact with the urogenital sinus epithelium. By days 12–14 the solid cord of cells is largely restricted to the mesonephros level, or slightly posterior to it, and is not usually evident in the genital cord. Throughout its course the Müllerian duct remains as a solid cord in the male, becoming progressively shorter until approximately day 50, when the anterior remnant reveals a definite lumen of 0.01–0.02 mm. in diameter. Subsequent to this period the short anterior remnant contains a definite lumen up to 0.03–0.04 mm. diameter, and in one case an expanded portion reached a diameter of 0.11 mm. The structure, therefore, shows definite growth in its anterior portion in normal males subsequent to day 50.

Table 1 indicates that 4 of the 13 males show evidence of androgenic stimulation of the Müllerian system. The greatest response occurs in animal 65A, sacrificed on day 39. The duct in normal males of this age is a solid nonluminate cord, whereas in this treated specimen a lumen was present which in one place was 0.09 mm. in diameter. Other males show definite, though less, stimulation than the male just cited. Figure 9 (Pl. V) (31-day male 64A, treated days 10–30) reveals the presence of a Müllerian duct with a small lumen that has been maintained over approximately its upper one-half. Since in normal 31-day males the duct is reduced to a solid nonluminate cord in its anterior portion, this case must be rated a response, even though of minimal proportions.

It is to be emphasized that androgens, though inducing some growth in size and the development of a lumen in Müllerian ducts in males, have failed to cause the ducts to grow in length or to prevent atrophy at their caudal ends. The youngest androgen-treated male (96A), sacrificed on day 16, reveals the Müllerian system to be solid degenerate cords extending approximately one-half the distance from the epididymis to the urogenital sinus. Thus, despite daily treatments from its first appearance, the usual postero-anterior retrogression has occurred, and a lumen was not established.

3. Testis responses: The testis responses to androgenic treatments have been relatively unimpressive; histologically, no outstanding or definite modification has been noted in these experiments at any age. In two cases attempts have been made to compare testis size in very young treated males with normal ones. Through utilization of the cutout-paper weight methods animal 47A, receiving testosterone ointment from day 3 to day 24, showed a total testis mass 30 per cent greater than that from a 24-day normal animal. Animal 64A, sacrificed on day 31, following injections of testosterone propionate on alternate days from day 10 to day 30, indicated a testis mass exceeding the normal control by as much as 81 per cent. These two estimates suggest increase in size of the testis at this age. At a later age, when the testis could be weighed immediately on removal and before fixation, this tendency did not continue. Thus, of two littermate males, sacrificed on day 83, the actual fresh weight of a single testis from an untreated male was 21 mg., whereas that of the treated was 24 mg. (treatment: 0.5 mg. *t*-propionate daily for 70–82 days). A second comparison between nonlittermates, but of the same body length and comparable weight, sacrificed on day 100, showed the normal testis weight to be 72 mg. and the treated testis 70 mg. (treatment 0.5 mg. *t*-propionate daily for 70–99 days). Two other animals sacrificed at an age estimated to be 1 year, and of comparable size, gave a weight of the two normal testes as 2.50 gm. and of the two treated testes as 2.40 gm. (treatment 5.0 mg. *t*-propionate daily for 33 days prior to sacrifice). Since histological study of all these cases revealed no characteristic or definite morphological differences in the treated organs, one must conclude that at these ages testosterone

and *t*-propionate in the dosages employed failed to cause perceptible modifications in the testis. It must be emphasized that the prostate gland, Cowper's gland, and phallus in these same animals revealed marked stimulation. The weight of the entire male reproductive tracts of the animals sacrificed on day 83, when compared, revealed that the reproductive system of the treated one was four times as heavy as that of the untreated normal; similarly, in the 100-day specimens the treated whole system was seven times the weight of the normal.

4. Prostate gland: The development of the prostate gland has been stimulated in all males treated with androgens and is perhaps second only to external genitalia in revealing responses to both early treatment and low dosage.

Chase (1939) has recently studied the normal prostate gland in the opossum, its origin and development, its final anatomy, and its response in adults to hormone manipulation. With the probable exception of such minor points as the state of development relative to embryonic age (my material being more abundant and more accurately timed) my observations offer confirmation of Mrs. Chase's account. Evidences of the prostate anlage as buds from the epithelium of the urogenital sinus appear on day 15. By day 32 (Pl. V, Fig. 10) prostate buds project from the entire surface of the anterior portion of the urogenital sinus epithelium, and by the 40th day the single bud outgrowths show beginning branchings permeating the surrounding mesenchyme. By the 100th day these branching outgrowths have laid the foundation for the final type of prostate gland possessed by this animal, but frequently canalization of the outgrowths has not occurred (see Pl. V, Fig. 14). In one normal male of 8 months the central ends of the outgrowths had become slightly luminate, with the outer two-thirds of the glands still solid epithelial cords; whereas in a second 8-month animal lumina extended to the peripheral terminations of the outgrowths, but the secretory cells were as yet quite below a functional level. In 3 males examined at an estimated age of 1 year the structure was assuming adult secretory characteristics.

As a means of representing responses of the prostate gland to androgenic treatments, Table 1 carries a list of measurements of the entire cross-section of the urogenital sinus and prostate outgrowths in comparison with similar aged normal animals shown in parentheses; this includes not alone the prostate outgrowth but the cavity of the urogenital sinus as well. Included in the table also is a subjective rating of prostate-gland response which is an attempt to portray comparative responses of the prostate outgrowths irrespective of growth in diameter of the entire urogenital sinus or its lumen. One can easily note that relative response of prostate-bud stimulation is not always parallel to increase in cross-section measurements. Animal 30A, despite a slight increase in total cross-section of urogenital sinus, reveals a prostate showing a high grade of precocious development. The response of the prostate of the 30-day-old treated male 101A (treated days 3–39) can be seen by comparing Figure 11 (Pl. V) with a normal 32-day-old male (Pl. V, Fig. 10); the comparison is direct by virtue of the same magnification.

Animal 64A, treated from day 10 to day 30 and sacrificed on day 31, was compared with an untreated 31-day male by means of weighing paper cutouts of the prostate tissue projecting from the surface of the urogenital sinus epithelium outlined at a magnification of 66×. Such weight comparisons of the prostate tissue in the two animals revealed that the prostate of the treated male was ten times that of the normal untreated prostate.

The variations in response in the treated series is naturally great, since sacrifice occurred at different ages and after different methods of application or different lengths of

treatment. Designations of comparative response noted in the table is an attempt to portray relative responses, and in each case judgment was made in comparisons between similar-aged treated and normal young.

5. Phallus: Phallic stimulation has occurred at all ages and with all dosages of androgens employed; in general, responses have been greater with advancing age. The phallus of untreated males grows slowly, since on day 16 measurements were 1.5×1.0 mm. and on day 44, 1.8×1.5 mm.; no untreated phallus up to 50 days of age gave greater measurements. All measurements of this structure were made from Bouin-preserved young in 80 per cent alcohol and hence are readily comparable. In comparison with these data from untreated males, it will be seen from the table that all males listed show a larger organ despite sacrifice of some treated specimens by day 16 subsequent to not more than 10 days of treatment. Enormous responses follow treatments begun on day 70 or later. In several cases the organ has assumed entirely adult proportions and morphology with the characteristic bifurcated glans.

In summary, treatment of male pouch young with androgenic substances results in strong stimulation of the prostate gland, Cowper's gland, and the phallus; mild stimulation of the Müllerian ducts; less evident stimulation of the Wolffian ducts; and little or no effects on the testes.

B. *Effects on females.*—The responses of the developing reproductive system of female pouch young to androgenic treatment have been studied in 23 serially sectioned, treated young, ranging in age at autopsy from day 16 to day 147. From this group 16 females have been chosen for tabulation of records of observations (Table 1) in such manner that the 11 youngest females could serve in comparison with littermate males simultaneously removed from the nipple for preservation following identical treatment; the older 5 females represent reactions at later ages, as well as results following discontinuance of treatment. The 7 females receiving no mention revealed entirely similar responses to those listed in the table; included among those listed are representatives from all types of androgenic treatment. In general, sensitivity to modification in development has followed in a descending order from phallus, prostate, Müllerian duct, Cowper's gland, Wolffian duct, to ovary (no appreciable response). Since Cowper's gland and ovarian responses will receive consideration elsewhere, they will be given but the briefest mention here.

1. Wolffian ducts: The youngest female (96B), sacrificed after androgenic treatment, was preserved on day 16, following daily treatments since day 3. Detectable modifications in Wolffian ducts were not apparent. Treatments of female 63B from day 10 to day 21 resulted in visible modifications on day 22. Normal 24-day female Wolffian ducts are decidedly degenerate. Connections with the urogenital sinus are represented by a solid cord of cells in the genital cord; more anteriorly the duct in the degenerate mesonephros, and slightly posterior, exhibits a barely discernible lumen. The ducts in treated female 63B on day 22 were provided with lumina from anterior levels to the region of the lateral vaginal canal; between the latter and the urogenital sinus it was a solid cord; anteriorly, the lumen had a diameter of 0.01 mm., but more posteriorly this attained a diameter of 0.04 mm., which compares favorably with the duct in a normal male of this age. The ducts in female 47B showed somewhat greater stimulation but also failed to show openings into the sinus.

Female 101B, treated with androgens between days 3 and 30, instead of showing the normal female Wolffian duct composed of a solid cord of cells and falling considerably

short of the urogenital sinus, contained luminate Wolffian ducts from the efferent tubule area, anteriorly, to the level of the lateral vaginal canals; luminal diameters of 0.03–0.04 mm. compare well with normal males of this age but in one area the experimental ducts reached a diameter of 0.11 mm. and failed to show entry into the sinus cavity. In treated female 64B, sacrificed on day 31, the Wolffian-duct outline can be seen faintly along the greatly enlarged Müllerian system (Pl. IV, Fig. 6). In sections (see Pl. V, Fig. 8) the Wolffian duct is seen as a luminate tube from the epoöphoron region to the level of the lateral vaginal canals; the luminal diameter is again approximately 0.04 mm. over its larger portion, but in the walls of the lateral vaginal canal it attains a diameter of 0.26 mm.

Female 66, treated with androgens between days 10 and 31, received no further treatment up to sacrifice on day 62. Despite the post-treatment recovery period, Wolffian ducts were luminate throughout, with the usual exception of the portion from the posterior part of the lateral vaginal canals to the urogenital sinus; this portion was reduced to a solid cord. The lumen anteriorly had a diameter of 0.03 mm. but along the uterine level this increased to 0.15 mm. and became greatly expanded along the walls of the lateral vaginal canals (0.75 mm.).

Female 117 received continuous treatment with androgens from day 3 to day 93; and, whereas the epoöphoron tubules were well developed, with luminal diameters up to 0.06 mm., the duct proper was reduced to a lumen of 0.02 mm., along side the uterus; in the region of the lateral vaginal canals the duct is further reduced to a nonluminate cord that can be traced to contact with the urogenital sinus epithelium. Female 118 was sacrificed at the same age but had gone without treatment between days 45 and 95, yet the Wolffian ducts were quite similar to those of female 117; in the lower uterine level a short segment was expanded to a luminal diameter of 0.37 mm. Female 120 was a littermate and received identical treatment to female 117 up to day 95; it was sacrificed without further treatment on day 147. Wolffian duct preservation differed in no remarkable way from female 117.

Generally speaking, the responses of the Wolffian ducts in females that had received continuous treatments with androgens, from a period much earlier than the beginning of duct regression following loss of urinary function, have not been striking. The epoöphoron tubules, fairly conspicuous in normal females of the opossum, revealed rather consistent responses, characterized by healthy preservation and increase in size. The more posterior portions of the ducts have given quite varied responses in the different treated females; but in all cases the ducts have been recognizable, usually with a small lumen, as far posterior as the lower levels of the lateral vaginal canals. Frequently, localized portions have become quite expanded, and such areas usually contain cellular debris. Only in female 107 was the connection with the urogenital sinus approximately normal for untreated males. Whereas bilateral preservation is usual, the posterior portions are quite characteristically reduced to solid epithelial structures in which a visible lumen is absent. Frequently along the lateral vaginal canals the ducts may be thrown into folds, as if a growth in length was stimulated. Androgens have preserved the ducts in females, therefore, but the preservation in most cases is atypical for normal males.

2. Müllerian duct: The stimulation of the Müllerian duct in female opossum pouch young from androgenic treatment has been a striking feature of this study, especially when comparisons are made with reactions of the same structure in treated males. All androgen-treated females have shown Müllerian-system stimulation, irrespective of

dosage, method of treatment, or age at sacrifice; it is true, however, that with advancing age or maturity of tissues the responses have been more pronounced.

The youngest female preserved after treatment (96B) was sacrificed on day 16 subsequent to daily application of testosterone ointment from day 3 to day 15. Normal females at this age have the Müllerian system represented by cords of cells continuous from the ostium to contact with the urogenital sinus; in occasional areas along the cord a tiny lumen is apparent, but for most of the distance the cord has not acquired a lumen. In this treated female a lumen is present throughout, though it is greater at the genital-cord level, where the diameter is 0.03 mm.; the lumen, however, is not traceable into the cavity of the urogenital sinus, as holds for all specimens under 100 days of age. The androgenic stimulation in this case, though definite, is minimal, as can be seen in the 22-day treated female.

Female 63B, sacrificed on day 22 subsequent to injections of *l*-propionate from day 10 to day 21, has been compared with a normal female of 24 days in order to attempt to portray the responses from androgenic treatment. In the normal 24-day female the various differentiated regions of the Müllerian duct have just become evident. The tube is essentially straight, with a lumen barely evident at the oviducal level but becoming more prominent at the uterine level (diameter 0.01 mm.), narrowing for the cervical region and expanding (diameter 0.01 mm.) for the lateral vaginal canal. When a comparison is made of this normal 24-day with the treated 22-day female, it is found that the normal oviduct lumen is barely perceptible, whereas treated oviducts are tortuous and show a luminal diameter of 0.03 mm. The normal uterus, with a total cross-section of 0.18 mm. and a lumen diameter of 0.01 mm., becomes in the treated female 0.87 mm. in total cross-section with a luminal diameter of 0.52 mm. Lateral vaginal canals of the normal have an entire cross-section of 0.18 mm. and a lumen of 0.01, whereas the treated lateral vaginal canals are 1.35 mm. in total cross-section and show a lumen of 1.10 mm Thus, the lumen of the treated uterus has a diameter approximately fifty times as great as the normal, and a lateral vaginal canal lumen one hundred times as great. The uterine epithelium of the treated female, while active, has not produced definite glandular outgrowths.

Treated female 64B, sacrificed on day 31 following *l*-propionate injections from day 10 to day 30, can be seen dissected to show the reproductive system in comparison with that of a normal 31-day female (Pl. IV, Figs. 5 and 6). From serial sections of these two female embryos one finds that the normal female uterus is 0.22 mm. in total cross-section and the lumen 0.07 mm. in diameter, whereas the uterus from the treated female is 1.65 mm. in total cross-section with a lumen 1.12 mm. Similar measurements for the lateral vaginal canals reveal the normal total cross-section to be 0.20 mm. and the lumen 0.06 mm., whereas in the treated female the total cross-section is 3.30 mm. and the lumen 3.00 mm. Rough comparison can be expressed by stating that the total uterine cross-section in the treated female is 8× normal and the lumen 16× normal, or the total lateral vaginal canal cross-section is 13× normal and the lumen 50× normal. In point of differentiation the treated uterus contains numerous well-formed glandular outgrowths which do not occur until several months of age in normal females. In female 64B the epithelium of the lateral vaginal canals has not advanced in differentiation to an extent comparable to uterine differentiation. Uteri of these two females, photographed at the same magnification, are shown in Figures 7 and 8 (Pl. V).

Such tremendous increases in the size of Müllerian ducts in the above case, and in

other treated females not described in detail, are frequently characterized by distention of the tube; it becomes evident that tissue growth has occurred, but it is similarly evident from microscopical study that differentiation has likewise been stimulated. Uterine glandular differentiation has advanced remarkably, and the mitotic index of many tissues is very high. To cite two examples, treated female 42 (not listed in the table) received testosterone-ointment treatment between days 16 and 50, with sacrifice on day 51. Caliper measurements of the lateral vaginal canal prior to fixation has been recorded as 6 mm. × 3 mm. When one examines the uterine glands in this female, the evident growth and differentiation of the uterus is revealed by numerous glands with an extremely high mitotic index; quite consistently, cross-sections of these glands reveal approximately each 8th to 10th nucleus in mitotic division—truly an extreme stimulation of mitosis in these nuclei of uterine glands. Female 47B, treated with testosterone ointment from day 3 to day 24 reveals moderate lateral vaginal canal stimulation (diameter 0.30 mm.; compare with treated female 63B, diameter 1.05 mm.); yet the mesenchyme cells (future muscle) surrounding the epithelium are extremely active. It is entirely consistent to see, within a single microscopic field (4-mm. objective), from 15 to 25 mesenchymal nuclei in mitotic division.

Tremendous responses to androgenic treatment characterize Müllerian reactions in all females, but older females differ from younger ones principally in greater increases in thickness of Müllerian duct walls and relative reduction in the lumen. This is due, to some extent, to the enormous glandular proliferations, more especially in the walls of the uterus than in the lateral vaginal canals. And, when once developed, the glands show but little involution after cessation of treatment, as may be seen in the case of the three older specimens listed in Table 1.

Females 117 and 118 were preserved on day 95, the former having received daily treatments from the third day until sacrifice, the latter having treatments discontinued on day 44, hence without treatment for the last 51 days. Figure 21 (Pl. VI) is a cross-section of the uterus of treated female 117, sacrificed on day 95, which should be compared with the uterus of a normal 100 day female (Pl. VI, Fig. 22). Since the figures are photomicrographs at the same magnification, comparison readily demonstrates the character of the response. The uterine wall of treated females is greatly thickened, glandular differentiation has been profuse, and the lumen of the tube is considerably reduced. Glandular formations are not indicated in the normal uterus at this age. The uterus of female 118 compares quite favorably with that of female 117, despite the fact that the former had received no treatment between days 45 and 95. Some glandular involution is apparent, but this is not great. In a similar manner the uterus of female 120 exhibits well-developed uterine glands despite the absence of treatment from day 96 to sacrifice on day 147.

3. Ovarian responses: The ovarian responses to androgenic substances will form the material for a separate account, and it will be indicated here only that marked modification in development has not been evident. By the utilization of projected sections on standard paper and of the weight of these sections, there is some indication that the mass of the ovary has been increased to a slight extent by androgenic treatments.

Histologically the opossum ovary presents many interesting problems, which will receive attention elsewhere, and it will be stated only in general that after any androgenic treatment the ovarian structure is not markedly modified. Its histological character appears thus far to have undergone some slight changes, but in any treated female ovarian

activity is evident in its various stages of organization. Older stages show well-formed normal appearing follicles, and the germinal epithelium at all stages of preservation appears active.

4. Prostate development: In females prostate development has not only been induced by treatment with androgens, but the degree of development and differentiation has also been surprising; normal females fail to develop the anlage of this structure at any stage. Such an animal as the rat may reveal prostatic homologues in the female in early development, only to have them regress and disappear entirely, or remain as rudimentary "paraurethral glands of Skene."

It is to be recalled that the prostate anlage in normal males appears on day 15. Despite its total absence in normal females, a treated female (96B) sacrificed on day 16, subsequent to 13 daily treatments with free testosterone ointment, showed prostate development of the characteristic male type, as well-formed buds growing out from the epithelium of the urogenital sinus; the extent of these outgrowths in this treated female is greater than in the normal male of similar age. Induction of a prostate by androgenic treatment, as well as precocious development, is here evident.

Prostatic responses in treated females are indicated in Table 1 by actual measurements of cross-sections of the urogenital sinus and prostatic outgrowths and, more adequately, by subjective ratings. Measurements, though reflecting increases in the total structure, do not differentiate between enlargements of the urogenital sinus cavity and the extent of actual prostatic outgrowths. Thus, in female 101B the diameter of the urogenital sinus and prostate is but slightly greater than the urogenital sinus diameter in a normal female of similar age; yet the response of the glandular outgrowths has been rated as "great stimulation."

A section of the urogenital sinus showing prostatic outgrowths in this treated female sacrificed on day 30 is shown in Figure 12 (Pl. V), photographed at the same magnification as is that of the normal 32-day male in Figure 10 (Pl. V). The much greater development in the treated female than in the normal male is evident; and if the comparison is made with the normal female, which shows no evidence of prostate outgrowths, the rating of "great stimulation" appears justified. The treated male prostate 101A, receiving identical treatment to its sister, shown in Figure 11 (Pl. V), although greater in amount, has received a lower subjective rating because the response has occurred in a male which would otherwise have had a prostate equivalent in development to Figure 10 (Pl. V). The grade of differentiation in the treated male prostate (Pl. V, Fig. 11) is approximately equivalent to a normal male of perhaps four months; that in the treated female prostate (Pl. V, Fig. 12) is equivalent approximately to 80–90 days of normal male development. It may appear that higher ratings are given to female prostate than to male prostate responses, but in each case the treated specimen is compared with a normal same-sexed individual and not with each other; female responses may, in a few cases, represent less total prostate tissue than in a similar aged male and yet receive a higher rating in relative response. In the 31-day treated female the prostate gland was compared by the paper-cutout method with that of a treated brother (64A) and a normal 31-day old male. In serial (10 μ) sections the total number of prostate sections in the normal male was 57; in the treated female, 131; and in the treated male, 153. By projecting prostate outgrowths at a magnification of 66× on standard paper and weighing the cutouts, it is revealed that the mass of prostate epithelium in the treated female is six times that of the normal male, while that of the treated brother is ten times the normal male.

Induced prostate development in treated females is not followed by immediate regression on cessation of treatment. Thus, females 118 and 120 each possess prostate glands rated "extreme stimulation" despite the absence of treatment during the last 50 days of life. Figure 15 (Pl. V) is a section of the urogenital sinus and prostate outgrowths from female 118 treated between days 3 and 44 and remaining without further treatment until sacrifice on day 95. When compared with Figure 14 (Pl. V), a section of the same region from a 100-day normal male, it is evident that the female prostate shows markedly greater differentiation despite the lack of treatment for the last 50 days of life.

5. Phallus: Phallic development in normal females is quite indistinguishable from that in normal males up to an age of approximately 30 days; in each case relatively slow growth occurs, and in normal animals measurements of the structure do not exceed 1.5×1.0 mm. On day 44 measurements of the phallus of the male were 1.8×1.5 mm. and for the female 1.5×1.0 mm. Thus, near the age of 50 days, phallic growth in the two sexes diverge, with the male showing more rapid subsequent growth.

The female phallus, like the male, exhibits marked growth responses to androgenic treatment. Attention to Table 1 reveals that the phallus of all treated females has responded by an increase in size over normal; the weaker response in females 63B and 38A has still produced organs larger than in normal females of like age. Also, the table shows that the phallus of littermates receiving identical treatment is larger in the male than in the female in 10 of the 11 pairs; female 101B had a phallus somewhat larger than the brother 101A. Treatment of females with androgens for longer periods, as in female 117, sacrificed on day 95, reveals the capacity of the female phallus to differentiate in the male direction. In this case the female phallus was considerably greater than in normal males of the age, and differentiation had rendered it a typical male phallus with bifurcate glans characteristic of the mature male organ. This clitoris, therefore, has undergone development entirely similar to that of the penis in males, and it compared favorably in size with that of a young mature male.

Since Burns (1939*d*, 1939*e*) has given considerable attention to phallic modification in opossum pouch young and since my materials confirm his main points, further details may be omitted. It is to be emphasized, however, that the phallus of the male shows somewhat greater growth responses than does that of the female. Furthermore, phallic modification appears to be one of the first to follow androgenic treatment, both in point of time and in point of response to low hormone dosage; the phallus and the prostate gland register the first of changes that follow this type of treatment.

C. *Comparative reactions of males and females to androgens.*—In the preceding pages attention has been directed largely to the effects of androgens on individual structures of males or females as these effects become evident from comparisons between the treated animal and the untreated normal one of the same sex and age. There appears to be some advantage in emphasizing the responses of the same structures to the same treatment in the two sexes. For this purpose it is of particular advantage to compare littermates sacrificed at the same time; this insures, as nearly as possible, a differential of sex only, since all members in a given pouch were treated exactly alike during the same period of development and with the same substance. For this purpose three pairs of pouch young will be compared at ages of 24-day, 30-day, and 39-day sacrifice, which well covers the period of heterosexual differentiation of the Wolffian and Müllerian systems. Entirely similar relations hold for all other pairs, as well as for isolated individuals from a pouch,

and in many cases even greater differences are shown than in these three pairs; one chief reason for the selection is the early age at which treatments were begun.

Twenty-four-day-pair: Pouch young 47A and 47B were sacrificed on day 24 subsequent to androgenic treatment daily from day 3 until the day of sacrifice. Wolffian-duct response in the male was essentially absent, but in the female (41B) it received a rating of "moderate stimulation." In a normal female the structure would have been reduced to a condition exhibiting the barest cavity for a lumen, and the solid cord posteriorly would have made an attachment with the urogenital-sinus epithelium. This treated female possesses a Wolffian duct with a lumen of 0.06 mm.; hence, in comparison with a normal female, it was stimulated. In point of relative comparison there is little difference in the duct at this age between a normal male and this treated male or female. Despite the evident stimulation in the female, therefore, the duct has been retained in essentially its typical state of development in normal males; in other words, regression of the structure in the treated female has been prevented.

Müllerian duct responses in the two sexes is materially different. Tabulated ratings for this pair show Müllerian ducts "slightly stimulated" in the male and "moderately stimulated" in the female, but the extreme difference between the end-products in the two sexes is not thus adequately represented. In normal males at 24 days Müllerian ducts are reduced to solid cords ending approximately one-half their length toward the urogenital sinus. Because of the development at anterior levels of a definite, though very small, lumen, slight stimulation over the condition in normal males is registered. As a general estimate, not more of the original duct persists than in the normal male, but the part which is present exhibits somewhat better development. In the normal female of 24 days the differentiated portions of the duct system would be barely indicated, and the lumen would have a gross diameter of 0.01 mm. The Müllerian system of the treated female, in exhibiting the definite formation of its various portions having lumina greatly in excess of the normal (oviduct, 0.03 mm.; uterus, 0.11 mm.; lateral vaginal canal, 0.30 mm.), has received a rating of "moderate stimulation." It must thus be evident that the Müllerian ducts of females respond to the same treatment with androgenic substances with an intensity entirely different than in males.

Thirty-day-pair: Comparisons of the duct responses to the same androgenic treatment in males and females sacrificed on day 30 may be made between pouch young 101A and 101B. These received daily treatments with androgenic material from day 3 until day 29.

The Wolffian ducts of the treated male (101A) were rated "slight stimulation" because the diameter of the ducts in the treated male was, in some areas, three times that of the normal control (0.09 and 0.03 mm). Luminal connections with the urogenital sinus had not been retained, and the usual solid cord termination was present. In the female (101B) "moderate stimulation" of the Wolffian duct was recorded. This is on account of the fact that in normal females of this age Wolffian ducts are reduced to solid cords of cells not reaching the urogenital sinus, whereas in this treated female the structure remained as a continuous tube with a luminal diameter of 0.03 to 0.11 mm. It did not continue posteriorly farther than in the normal female, but the portion remaining had been converted into a definite luminate tube. Thus, despite androgenic treatments since day 3, the full length of the duct was not retained.

Müllerian ducts exhibit an immense difference in the two sexes receiving the same hormonal treatment. In normal males of this age the Müllerian system would have been re-

duced to a solid cord extending approximately one-half the distance to the urogenital sinus. In the treated male it was present over approximately the same distance, but anteriorly it possessed a lumen of 0.01 mm., hence the rating "slight stimulation." In normal females the duct would have shown a maximal luminal diameter of 0.03 mm., and this would have been at the lateral-vaginal-canal level. The treated female exhibited a luminal diameter of this portion of 1.42 mm., or approximately forty-five times normal, and a uterine diameter of from seven to ten times normal; the uterus contained well-developed glandular formations; hence the rating of "great stimulation" was recorded. But consideration of the Müllerian response in the male, as compared with the female, emphasizes that the comparison is between a tube one-half its length, having a lumen of 0.01 mm., and a well-differentiated complete female system having a maximum lumen of more than one hundred times the male. It is to be emphasized at this point, therefore, that Wolffian ducts, though responding modestly, are in the end approximately equal in the two sexes, whereas Müllerian duct responses in the two sexes show tremendous discrepancies despite treatments with androgens begun at an early age.

Thirty-nine-day-pair: Pouch young 65A and 65B are the oldest heterosexual pair of littermates to be sacrificed on the same day from the same pouch and therefore having had identical treatment. Many older males and females from different pouches are available, but the advantage in utilizing littermates for comparative duct reactions will be evident.

Wolffian duct response in 65A was rated "slight stimulation" because the duct lumen was approximately twice that of normal male controls (0.06 mm. and 0.03 mm.). No other evident signs of stimulation of the duct were present with the exception that efferent tubules were apparently stimulated to some extent. In the female 65B "slight stimulation" was registered because in normal control animals this structure would have been a severely reduced solid cord of cells ending anterior to the genital cord. In the treated female it existed as a solid cord of cells still connected with the urogenital sinus, and throughout its course showed a lumen of 0.03–.06 mm.; a distended portion opposite the lateral vaginal canals possessed a luminal diameter of 0.37 mm.

Müllerian ducts again exhibit tremendous discrepancies in their responses in the two sexes. In the treated male the structure has been retained in approximately one-half its original length and anteriorly exhibits a lumen of 0.03 mm. diameter. The stimulation is therefore evident. In the normal female of this age the system would have possessed a maximal luminal diameter of probably 0.20 mm. for the uterus and 0.30 mm. for the lateral-vaginal-canal portion. This treated female had a uterus with well-developed glands and a lumen of 1.5 mm. and a lateral-vaginal-canal portion of 1.6 mm.

This tremendous difference in the Müllerian system of a male and female treated with androgenic substances since the 10th day, at which time Müllerian ducts in the two sexes are essentially alike and have just reached the urogenital-sinus area, clearly shows that this structure in the two sexes exhibits differential responses; the male Müllerian ducts and those of the female do not have the same capacities to respond to this hormonal treatment.

In a résumé of the effects of androgens on the early sexual differential of pouch-young opossums it may be briefly recalled that (*a*) the gonads of either males or females are not markedly affected; (*b*) Wolffian ducts of both sexes show only minor responses to androgenic treatment, and the structures are maintained in part or fairly complete in females; (*c*) Müllerian ducts respond in each sex but in a tremendously different manner; (*d*) pros-

tate glands are markedly stimulated in both sexes, but induction in females occurs before the great growth responses can occur; (*e*) Cowper's gland in both sexes responds markedly; (*f*) the phallus is greatly stimulated in each sex, slightly more in males, and that of females is modified to the typical male type.

V. EFFECT OF ESTROGENIC TREATMENT

Estrogenic substances have been found to be decidedly toxic for pouch-young opossums. Such treatments have involved the subcutaneous injections of theelin in oil, injections of estradiol (progynon-B) in oil, estradiol in alcoholic solution applied to the skin surface, and estradiol in ointment form. Earlier treatments were largely fatal to embryos, probably because of too high dosage.

The majority of the pouch young have been treated by the application of a commercial face cream to the surface of the skin by means of a small brush. The face cream was purchased over the counter of a leading Chicago department store and was recommended to women as a preventive for facial wrinkles and a general skin beautifier. It contained 0.625 mg. of estradiol per ounce of cream, and its estrogenic properties and skin penetrability were rather thoroughly tested on adult rats and guinea pigs (Moore, Lamar, and Beck, 1938). Its capacity to produce, in very small quantities, cornified vaginal smears in spayed rats, uterine growth after ovariectomy, mammary-gland growth in male guinea pigs, and testicular injury in normal male rats are entirely in keeping with similar phenomena obtained through injections of estradiol in oil.

Ointment application is very difficult to quantitate, because of the fact that similar thin body coverings will require progressively larger amounts of the cream as the animals grow, and also because of the fact that pouch young are not only in contact with each other but also in contact with the mother's pouch. One is entirely unable to state approximate amounts of the hormone absorbed, but the amounts applied daily would be of the approximate order of 1 γ daily for younger stages and not over 6 γ for the older and larger pouch young.

With this type of treatment there was still a large loss of pouch young. Treatments of 5-day-old pouch young were often followed by a daily loss of 1–4 young from the pouch, and all members of a pouch may be lost before treatments have been given for 5 days. Approximately 10 litters have been started on treatment, and a typical example is the final preservation of 4 pouch young from a pouch in which treatments were begun on 12. In a few cases young were recovered from the cage before death occurred, and all dead young recovered have been preserved; some of these were fairly satisfactory for study. Consideration of effects will be restricted to young which were removed from the nipple at desired periods and to a few discovered while still alive but which had become detached from the nipple. Owing to the delicacy of younger individuals, 1 or 2 hours out of the pouch is fatal. All pouches were examined at 24-hour intervals during the course of treatments, and in many cases they were examined more frequently; despite this fact, many dead ones were unfit for study. The group to be considered here consists of 8 males and 11 females.

A. *Males.*—In Table 2 are to be found essential data on 8 male opossum pouch young receiving estrogenic treatment; these data include ages at beginning and termination of treatment, as well as brief notations representing responses of the reproductive system. Four males of the group represent treatments started at ages prior to completion of back-

ward growth of Müllerian ducts toward the urogenital sinus, 2 started at ages subsequent to such contact, and 2 at periods approaching the time of detachment from nipples.

Ser. 97: The youngest male preserved, Ser. 97, as well as the one receiving the shortest treatment, received the first application of estrogenic ointment on day 6, when Müllerian ducts were represented by solid cords of cells extending approximately to the caudal end of the mesonephroi: it was preserved on day 12, 1 day after the last of 6

TABLE 2

EFFECTS OF ESTROGENS ON OPOSSUM POUCH YOUNG*

Ser. No.	Age at Death (Days)	Treatment (Days)	Wolffian Duct	Müllerian Duct (Mm.)				Phallus
				Oviduct	Uterus	Cervix	Lateral Vaginal Canal	
			Males					
97	12	6–11	0.06 (0.04)	Cord	Cord	.....	.....	1.5×1.0
49A	17	5–16	0.22 (0.06)	.....	0.15 (0.00)	.....	.....	2.0×2.0
59	17	10–16	0.07 (0.06)	.....	0.15 (0.00)	.....	.....	1.7×1.0
55A	23	5–22	0.60 (0.04)	.....	0.22 (0.00)	.....	.....	1.2×1.5
109	30	6–29	0.15 (0.03)	.....	0.22 (0.00)	.....	.....	3.5×3.0
39A	31	17–30	0.06 (0.03)	.....	0.07 (0.00)	.....	.....	1.5×1.3
93	83	70–82	0.07 (0.03)	.....	0.33 (0.03)	.....	.....	.....
95	88	70–87	0.07 (0.04)	.....	0.52 (0.03)	.....	.....	.....
			Females					
49B	17	5–16	0.90 (0.06)	0.01 (0.00)	0.30 (0.00)	.....	.....	2.0×2.0
60	19	10–18	0.33 (0.06)	0.01 (0.00)	0.18 (0.00)	.....	.....	2.0×1.2
100	22	6–21	0.60 (0.00)	0.01 (0.00)	0.45 (0.01)	0.07 (0.00)	1.35 (0.00)	4.1×3.0
55B	23	5–22	0.45 (0.00)	0.02 (0.00)	0.33 (0.01)	0.07 (0.00)	0.15 (0.01)	1.6×1.6
108	30	6–29	2.15 (0.00)	0.03 (0.01)	2.85 (0.02)	0.07	2.15 (0.03)	4.0×2.0
106	35	6–34	1.30 (0.00)	0.04 (0.03)	1.80 (0.07)	0.09 (0.03)	1.80 (0.11)	6.0×5.0
114	39	6–38	0.82 (0.00)	0.03 (0.03)	3.00 (0.30)	0.07 (0.07)	0.70 (0.37)	4.0×4.0
110	43	6–42	2.10 (0.00)	0.03 (0.03)	2.70 (0.30)	0.07 (0.07)	1.70 (0.37)	4.0×3.5
111	46	6–45	1.50 (0.00)	0.03 (0.03)	4.50 (0.30)	0.07	1.20 (0.37)	5.0×2.0
115	46	6–45	1.80 (0.00)	0.03	2.85 (0.30)	0.07	1.45 (0.37)	4.5×4.0
119	79	6–45 46–79 no tr.	0.75	0.04	1.80 (0.65)	0.07	1.50 (0.75)	4.1×1.9

* Greatest diameters of lumina of ducts in millimeters; ocular micrometer measurements from fixed preparations. Figures in parentheses show diameter of lumen in normal of same age and sex. Phallus, millimeters.

treatments, at an age when Müllerian ducts would have approached contact with urogenital-sinus epithelium. The treatments were apparently without demonstrable effects. Wolffian ducts were normal in caliber, and Müllerian ducts were represented by cords of cells just approaching the urogenital-sinus epithelium; no growth stimulus was apparent in these ducts.

Ser. 49A: This 17-day-old treated male shows modification. Wolffian ducts in 4 untreated males on the 16th day revealed patent ducts throughout their course with a

luminal diameter not exceeding 0.06 mm.; and, in each of these young, connections of the lumen with the cavity of the urogenital sinus were still perceptible. In this male, treated with estogens by ointment, the Wolffian duct likewise entered the urogenital sinus, but it was sufficiently enlarged along its course to show a maximal luminal diameter of 0.22 mm. (over 3× normal). Müllerian ducts, which in normal males were reduced to solid cords and terminated anterior to the genital cord, were large luminate ducts. Anteriorly an ostium was not seen. The duct, in its course through the mesonephros region, contained a well-defined lumen which more posteriorly increased to a diameter of 0.15 mm. At the posterior end the ducts ended blindly considerably anterior to the urogenital sinus. This region itself exhibited a tremendous hyperplasia and a central sloughing of its innermost layers of cells.

A prostate gland, visible in all four 16-day-old control males, was not in evidence in this 17-day-old treated male.

Ser. 55A. Marked response to estrogenic treatment was given by a male sacrificed on day 23. At this age normal males show marked reduction in Wolffian ducts, subsequent to loss of mesonephros function; a Müllerian duct represented by a solid cord of cells terminating considerably anterior to the urogenital sinus; and a prostate gland well represented by outgrowths from the urogenital-sinus epithelium.

This treated male had Wolffian ducts showing patency throughout their course and containing considerable cellular debris in the lumen. The lumen of the duct was small anteriorly in the substance of the mesonephros (luminal diameter 0.07 mm.), but more posteriorly it was enlarged to a luminal diameter of 0.60 mm. (20× normal). The lumen was traceable into the uppermost part of the urogenital-sinus horn, but hyperplasia was so great that the actual luminal connection with the latter cavity is somewhat questionable.

Müllerian ducts terminated blindly at both ends; an ostium was not present; and the segment representing the oviducal level was composed of isolated, disconnected short luminate pieces. In the caudal portion of the degenerating mesonephros the duct became continuous, and posteriorly it terminated quite anterior to the genital cord and attained a luminal diameter of 0.22 mm. Figure 18 (Pl. VI) is a section through this estrogen-treated male, which should be compared with the androgen-treated male 64A (Pl. V, Fig. 9) for relative duct responses.

The prostate gland was not visible. The entire urogenital sinus was a very large solid mass of cells peripherally, and centrally numerous layers of dying cells were being sloughed into the lumen; the hyperplasia was pronounced. This type of response of the prostate region may be seen in Figure 13 (Pl. V), which is a section of estrogen-treated male 39A, sacrificed on day 31.

Ser. 93–95: Representing treatment on older males, series 93–95 are particularly interesting because of effects on the prostate gland. Daily subcutaneous injections of 20 I.U. progynon-B were begun on day 70. Sacrifice of series 93, along with an untreated male control, occurred on day 83, and it was anticipated that treatment would continue on series 95 until day 100; on day 87, however, this male was perceptibly edematous, and the reproductive tract was removed shortly after death on day 88. The following brief remarks refer to series 93, sacrificed on day 83.

The entire reproductive tract was preserved in an extended position in Bouin's fluid; and, after transfer to 80 per cent alcohol, the weight of the entire tract (minus bladder, and one testis otherwise preserved) was 0.3842 gm., while that of the normal control was

0.1148 gm. Thus, despite a total body weight for the untreated male (72 gm.) greater than that of the estradiol-treated male (56 gm.), the reproductive tract of the latter weighed more than 3× that of the normal. The prostate-gland area of the treated male was much larger in gross size, and at comparable levels the diameter of an entire cross-section was approximately three times that of the normal control. Histological sections of this stimulated prostatic region show clearly that, whereas estrogens impart a tremendous growth stimulus to this area, the reaction is not one of prostate differentiation but a tremendous hyperplasia and metaplasia of the epithelial elements. Figure 16 (Pl. V), illustrating the reaction, should be compared with Figure 14 (Pl. V), a section of a 100-day normal male prostate and urogenital sinus. It is clear that a marked hyperplasia, involving the branching cords of prostate epithelium, has been induced and that the entire extent of the prostate cords is involved. The reaction is similar to responses of the urogenital-sinus epithelium to estrogenic treatment of the 31-day male shown in Figure 13 (Pl. V). This typical hyperplasia and metaplasia has followed estrogenic treatments in males, as well as in females, and no sexual difference in the response of the urogenital sinus has followed such treatment except in the case of males, in which treatment was begun after prostate development had advanced. In early stages, therefore, all prostate development in males is suppressed, whereas in later stages the outgrowths are markedly modified.

Testis development, or testis weight, in comparison with that of the normal male, was not modified during the course of 13 days of treatment; weight and histological character reveal no perceptible change.

Wolffian ducts, represented by the vas deferens and epididymal tubules, showed an increase in luminal diameter which was approximately double that of the normal; and the remnants of Müllerian ducts, representing a blind persistent remnant along the course of the epididymis, showed great increase in size as indicated in the table.

In general, estrogenic treatments of developing males, especially prior to day 30, have resulted in stimulation of both Wolffian and Müllerian ducts, the intensity of stimulation for both ducts being lower than that for the same ducts in females. The responses of Müllerian ducts in males to estrogens might have been anticipated, but the character of response may be unexpected. The 6 males receiving treatment during the period of caudal growth of these ducts failed to demonstrate the capabilities of the hormone in promoting more rapid growth in this direction or of retaining the portion of the duct which already had developed; despite the treatments, the ducts regressed in a postero-anterior direction and ended blindly. The responses, therefore, are essentially restricted to an increase in size of those remnants that have been maintained. Anteriorly the ducts are not retained; ostium development shows no stimulation, but rather regression; and the oviducal portion is largely absent despite continuous estrogenic treatment. Stimulation is shown from the fact that, at ages when Müllerian ducts in normal males would be reduced to solid cords of cells, these ducts in treated males were relatively large luminate ducts lined with a good epithelium; but these growth-stimulating phenomena are not in a direction of normal morphological differentiation; glandular developments are not evident. Wolffian ducts, likewise, demonstrated some increase in size, but this becomes less evident with increasing age.

Effects of estrogenic substances on the urogenital sinus have been principally the induction of a distinct and pronounced hyperplasia, with proliferation of cells proceeding centrally and with sloughing of cornified-type layers into the lumen. The germinative

layer is always on the outside; and, instead of the normal prostatic outgrowths in the form of cords of cells proceeding peripherally, all cell movement has been directed centrally—a type of polarity reversal, as far as the growth tendencies of the epithelium are concerned. Furthermore, prostatic outgrowths, well formed prior to the beginning of treatment, are themselves subject to hyperplasia and to metaplasia, with a conversion of the orderly processes in development into a more or less unorganized, riotous, development of masses of relatively undifferentiated cells.

B. *Females.*—In Table 2 is a list of 11 females, subjected to estrogenic treatment for different total periods and at different ages, that will receive consideration here. As a measure of response the diameter of the lumen of the ducts, as well as the different segments of the Müllerian ducts, are listed as determined from sections by an ocular micrometer; frequently, outside diameters are ill defined because of contiguous structures, but, even though more accurately determined, luminal diameters fail to show thickness of walls of the structures listed. Treatments on the majority of these females were begun on day 5 or 6, since at this period Müllerian ducts are in process of formation; they are growing caudally but will not yet have reached contact with the urogenital sinus.

Detailed consideration will be restricted to females 49B and 115, representing the youngest and oldest continuously treated individuals of the group.

Ser. 49B: This female received daily treatments with estradiol ointment from day 5 to day 16 and was removed from the nipple on day 17. On this day a normal female possesses a Wolffian duct, into which open the mesonephric tubules, and the duct possesses a well-defined lumen extending to the urogenital sinus, but the actual luminal entrance may be barely interrupted: its greatest lumen diameter is 0.05–0.06 mm. The Müllerian duct barely, if actually, contacts the urogenital sinus, and throughout its course from the ostium posteriorly there will be no regional differentiation; simple columnar epithelium will, for the most part, fail to reveal a lumen, but in places the lumen will be evident.

This treated female, in contrast to the normal, reveals a tremendously stimulated set of ducts. The Wolffian duct is complete from the efferent tubule area anteriorly to definite contact with the urogenital-sinus horn. Anteriorly the lumen is 0.11 mm., but caudally it gradually increases in diameter to 0.37 mm. in the uterine area; and just anterior to contact with the urogenital sinus the lumen reaches a diameter of 0.90 mm.—a diameter of more than fifteen times that of the Wolffian duct in a normal female of the same age. Considerable cellular debris is present in the duct, some of which may represent degenerating kidney material, some from cells possibly cast off from the Wolffian duct wall and from other cells that may have sloughed from the greatly stimulated urogenital sinus. Figure 19 (Pl. VI) is a cross-section through the ducts of the 23-day treated female, 55B, and may be compared with sections of a normal 31-day female (Pl. V, Fig. 7) or the androgen-treated male in Figure 9 (Pl. V). The urogenital-sinus epithelium has experienced a tremendous hyperplasia leading to large solid cellular masses and broken down, cornified, epithelium sloughing into its cavity (similar to Pl. V, Fig. 13).

The Müllerian duct, which in a similar-aged normal female would be solid, or barely revealing a lumen, is differentiated in the treated female into an oviducal portion (lumen 0.015 mm.) and a well-developed uterine region divided into internal partitions from foldings, suggesting beginning glandular formations; the maximal lumen diameter of 0.37 mm. represents an increase of approximately forty times that in the untreated normal female. The duct becomes narrower, and shortly caudal to a point where the

luminal diameter is 0.06 mm. it ends blindly without clearly contacting the urogenital-sinus epithelium; no lateral vaginal canals are clearly indicated.

The phenominal response of the urogenital-sinus epithelium is characterized by great hyperplasia and by an internal cornification and sloughing of epithelium into the lumen, as well as great extensions in development of the sinus horns which are represented by large solid masses of epithelial cells. A cross-section through the urogenital-sinus region shows the diameter of a cross-section to be nine times that of an untreated female of similar age.

Ser. 115: This female was sacrificed on day 46 after daily treatments with estradiol ointment from day 6 to day 45; it is one of the longer-treated females but is chosen especially for comparison with series 119. These two females, in the same pouch, received identical treatments to day 45; one was preserved to indicate the stage of development 1 day after the last treatment, whereas the second one was permitted to survive without further treatment until day 79.

The condition of female 115 is that of tremendous stimulation of both Wolffian and Müllerian ducts. The normal untreated female of this age shows the Wolffian duct greatly reduced; anteriorly, epoöphoron structures are mostly solid epithelial cords traceable to the rete ovarii. The duct is visible for approximately one-half the distance to the urogenital sinus and is reduced to a solid cord, gradually undergoing atrophy. In contrast, treated female 115 possesses a Wolffian duct which in the anterior region has a well-defined lumen (0.03 mm. diameter); epoöphoron tubules are but little stimulated. Traced caudally, the duct becomes greatly enlarged in size and in the uterine-segment level the Wolffian lumen reaches a diameter of 1.50 mm. and nearer the urogenital-sinus area a diameter of 1.80 mm. Figure 17 (Pl. VI) shows the Wolffian duct imbedded in the walls of the uterus at a level where the diameter of its lumen is 0.2 mm. The duct contains considerable cellular debris, and posteriorly the lumen passes over into the urogenital-sinus region with its tremendous hyperplasia and cornified cell reaction. Thus, not only has estrogenic treatment resulted in the preservation of the Wolffian duct throughout its course, but it has also resulted in causing sufficient growth to produce a lumen of 1.80 mm. in a region in which the duct is totally absent in normal females.

Müllerian ducts in normal females of this age show regional differentiation into an oviduct that is slightly tortuous (lumen 0.03 mm.), a uterine portion somewhat expanded but still lined with a simple epithelium with no evidence of glandular formations (lumen diameter 0.30 mm.), a constricted cervical region (0.07 mm.), and a slightly expanded region for the lateral vaginal canal (0.37 mm), which comes in contact with a solid plug of cells at the urogenital sinus.

The treated female, in contrast, shows tremendous enlargements for the uterine and lateral-vaginal-canal portions but little change in the oviduct portion (aside from more convolutions) or in the constricted cervical canal portion. The uterine portion, despite a growth sufficient to produce a lumen of 2.85 mm. (nine times normal), shows well-developed areas of uterine glands (Pl. VI, Fig. 17); in some areas of the duct, pressures have apparently effectively flattened the epithelium and prevented glandular outgrowths. Lateral vaginal canals are enlarged to a diameter of 2.10 mm. (six times normal), and these open into the greatly hypertrophied urogenital-sinus area. Figure 20 (Pl. VI) shows the transition from the glandular epithelium of the lateral vaginal canal to the hyperplastic urogenital-sinus area, much more apparent at a different level than in this section. Female 119 received identical treatment except that it was not sacrificed until day 79 and

hence was without treatment after day 45. Whereas some regression in size of the ducts may have occurred, they are still of essentially the same grade of development as in treated female 115.

In general, such responses as have been described for this youngest and oldest female among the treated group portray the general nature of the modifications produced in young developing females after daily treatments with estrogenic substances during sexual differentiation. Slight individual differences naturally occur, but the notations of lumen diameters in the table roughly express some of these variabilities.

The Wolffian ducts in treated females have, without exception, shown responses not only by their presence at ages when normals have regressed but also by the tremendous growth responses required to produce ducts with lumina up to 2 mm. in diameter in sectioned materials. Frequently a duct on one side may be much greater than the opposite, but no selective laterality in response has been noted. Considerable differences exist also in the amount of cellular debris present in the lumen. Stimulation of the epoöphoron region has not been conspicuous, but decided stimulation has been evident in posterior mesonephric tubules (paraoöphoron) in females at ages when normally these would be reduced to solid degenerate remnants. In older females, in particular, the caudal end of the duct is frequently seen to be thrown into pronounced folds, exhibiting considerable grades of metaplasia, as if linear growth tendencies have been unable to express themselves.

Müllerian ducts in estrogen-treated females have responded invariably with pronounced growth; this has involved precocious regional differentiation, precocious glandular developments, and great increase in size. Inherent regional tendencies express themselves as they would at later ages in untreated normal animals, since, if treatments were initiated prior to the Müllerian duct contact with the urogenital sinus, the simple tube still responds with a distinct oviducal and uterine portion. The two youngest females listed in Table 2 did not show distinctly a lateral vaginal canal region, but by the 22d day (ser. 100) this was well indicated, and the constricted cervix portion connected the uterus and lateral vaginal canal.

Casual study of ovaries has failed to reveal outstanding modifications, but normal ovarian development and hormonal responses of this organ are being investigated by Mr. Charles Morgan.

For the purpose of contrasting differential responses in the two sexes to treatments with estrogenic substances, it is desired to call attention to three pairs of embryos representing a male and a female, in the same pouch, that had received identical treatments.

Male 49A and female 49B were treated on days 5–16 and sacrificed on day 17. It can be seen from Table 2 that Wolffian ducts in the female were approximately four times as large as those in the male and that female Müllerian ducts were approximately double those in the male. Male 55A and female 55B which were treated from day 5 to day 22 and sacrificed on day 23 show that the Wolffian ducts were actually somewhat smaller in the male than in the female, but the Müllerian ducts in the female were again much more responsive than those in the male. The pair, male 109 and female 108, treated from day 6 to day 29 and sacrificed on day 30, reveal Wolffian ducts in the female approximately fifteen times the diameter of those in the male and Müllerian ducts approximately thirteen times greater in luminal diameter in the female. This selective tendency of both sets of ducts in females to respond more vigorously than in males in the same pouch and

receiving identical treatments is further emphasized by a glance at the measurements recorded in the table for the group as a whole.

From such comparisons it becomes evident that the homologous ducts respond differently in the two sexes to the same treatment. The genetic constitution appears to establish a foundation for differential reactivity to the same chemical treatment, even though treatments are given continuously from a period prior to definite embryological formation of the ducts.

In résumé, estrogenic treatments of developing opossums have induced growth in external genitalia of both sexes, but the phallus differs qualitatively from that produced by androgens. Wolffian ducts have been stimulated in both sexes, but more intensely in females; they have been preserved from disappearance and increased greatly in size. Müllerian ducts have been stimulated in both sexes, moderately in males, tremendously in females; in males ostium and oviduct are usually not preserved, and the tube is blind at both ends, while in females precocious regional differentiation, general growth, and glandular differentiation have been evident. The prostate gland in males is suppressed or, if already formed, is converted into an atypical hyperplastic mass. The urogenital sinus is very sensitive to estrogenic effects and undergoes tremendous hyperplasia and metaplasia. Gonads are not markedly modified.

VI. ADMINISTRATION OF GONADOTROPIC SUBSTANCES

The gonad-stimulating principle of pregnant mare serum (equine gonadotropin) has been administered to both male and female opossums at different periods in development. Provided the gonads would respond by precocious or accelerated sex-hormones secretion, the results on the various end-organs should be most instructive in comparison with responses of the same organs to the chemical hormones that have been applied from without. Furthermore, since the gonads of many species are remarkably stimulated to excess hormone secretion, especially after the attainment of a definite stage in maturity, it was anticipated that perhaps some suggestion might follow with regard to the age at which gonads first begin secreting their respective hormones, or at least when they were definitely capable of doing so. If this should prove to be the case, we would be provided with a means by which to determine the onset of secretion under normal conditions.

This phase of the study must be regarded as the most incomplete, since the number of developing young available for this purpose was small. Table 3 gives a list of 9 individuals which received treatment, recording age, type of hormone, and duration of the injection period. Gonadogen and gonadin are each the concentrated gonadotropic principle from pregnancy serum. Daily injections of 1 rat unit of gonadogen into rats for a 17-day period, terminating with a body weight of 60 gm., revealed a thirty-times increase in fresh weight of seminal vesicles over normal controls; similar daily injections of 5 rat units of gonadin (different unitage) produced an increase of twenty to twenty-five times in seminal-vesicle weight. In 30-day female rats similar dosages of each preparation produced an increase in the fresh weight of the uterus approximately ten times that of the control female. The body weight of treated opossums was in the range of 3–4 gm. at the end of treatment for younger stages and of 130 gm. for the 100-day specimens: excessive amounts of these hormones were thus given younger opossums.

A. *Males.*—No effects from the treatment were perceptible in the 17-day and 21-day males. Careful examination of testes Wolffian ducts, Müllerian ducts, prostate gland,

and urogenital sinus, in comparison with similar aged normal males, reveals no detectable modification. In the 30-day male (ser. 112) prostate-gland and Cowper's-gland differentiation on a subjective basis appeared to be slightly stimulated; actual microscopical measurements, however, failed to reveal any very evident increases in total cross-section of the urogenital sinus and prostate outgrowths, although the length attained by prostate cords appears to be slightly greater than in a similar-aged normal control. Müllerian ducts or Wolffian ducts were not perceptibly modified. If this is really a case of response, as it appears to be, the stimulation is but very slight. The 100-day male (ser. 84) reveals definite, though slight, acceleration in prostate differentiation. The central ends of prostate cords, at their attachment to the urogenital-sinus epithelium, show more definite luminal formation than in the control. Differentiation, however, has not ap-

TABLE 3

GONADOTROPIC TREATMENT OF OPOSSUM POUCH YOUNG

Ser.	Age at Autopsy (Days)	Daily Treatment
	Males	
99A	17	10–16 gonadogen, 1 rat unit
102A	21	10–20 gonadogen, 1 rat unit
112	30	15–29 gonadin, 5 rat units
84	100	85–99 gonadogen, 5 rat units
	Females	
99B	17	10–16 gonadogen, 1 rat unit
102B	21	10–20 gonadogen, 1 rat units
113	30	15–29 gonadin, 5 rat units
116	38	15–37 gonadin, 5 rat units
90	100	85–99 gonadogen, 5 rat units

proached the stage of puberal development. The weight of one testicle of this treated male, in comparison with an untreated littermate, was approximately 60 per cent heavier, but there is no apparent histological difference between the two organs.

Gonadotropic treatments have given but very slight evidences of effectiveness in stimulating the testicle of developing male opossums to secrete hormones; and until other material is available for study, the limitations of effectiveness will have to remain uncertain.

B. *Females.*—A study of the 5 treated females listed in Table 3 is summarized by the statement that no effects have been observed from the treatments given. Younger specimens carefully examined for Müllerian and Wolffian duct and urogenital-sinus modification have revealed no responses. Even in the 100-day treated specimen a study of the ovary fails to elicit any detectable response; no suggestion of antrum formation in follicles is evident.

VII. DISCUSSION

The study which has been conducted on the development of the reproductive system of the opossum, and the unusual aspects of it that follow treatment with sex hormones, has involved several somewhat special questions, some of which may be formulated as follows: (1) Does the differentiating reproductive system respond to treatments with androgenic, estrogenic, or gonadotropic substances, and, if so, how early do such effects become apparent? (2) Can artificially introduced chemical hormones effectively override zygotic sex-differentiating factors? (3) Is there specificity in response of developing structures to these chemical substances—male structures being stimulated by androgens and female structures by estrogens? (4) What bearing do observations have on the question of hormone antagonism? (5) How do effects from such treatments with chemical hormones compare with results noted in freemartins? (6) From the nature of responses, if any, what suggestions appear relative to the possible role of such substances in normal sex differentiation, or the possible part played by hormones secreted by developing gonads? Additional supplementary questions have presented themselves, but the essence of the inquiry is perhaps sufficiently laid before us for consideration.

1. Attention to the preceding pages can leave no doubt that both the developing male and female reproductive systems are subject to modifications by application of pure chemical androgens or estrogens. In point of time, responses can occur early, since at the 15th or 16th day in the pouch, after 11 days of treatment, some modifications have become evident. At later periods in development and with fewer days of treatment, much more marked modifications are apparent, which suggests that a certain degree of differentiation is required before responses occur; to determine at just what point, in terms of maturity of the developing system, the ducts become sensitive requires additional experimentation.

In point of character, the responses have all been of the nature of stimulations rather than inhibitions, and no clear instance of inhibitory phenomena has been apparent. One could perhaps speak of prostate-gland inhibition in males by estrogens, since under such treatments the prostatic outgrowths from the urogenital-sinus epithelium fail to become evident. However, the actual response of this region is characterized by great stimulation of cell division, and hyperplasia, in the urogenital-sinus epithelium; and it appears more accurate to regard the end-result as an example of competition between two different types of stimulated cell response—peripheral cellular growth for prostatic cord formation, and proliferation centrally, giving the high degree of hyperplasia and accompanying metaplasia; certainly, the total amount of epithelium is much increased over normal when estrogens are applied, hence inhibition could only be held for the *type of differentiation*. The region, and actual source of prostate cells, has been greatly stimulated and not inhibited, even though the course of development has been turned in a different direction.

A second modification capable of being interpreted as inhibition is that of the phallus after estrogenic treatment. But here again, as with the prostate, there has been real stimulation of this structure and not an inhibition; the total mass has been markedly increased, but its development has followed a different course from that dictated by its genetic constitution.

Since these two mentioned conditions are thus interpreted as stimulations rather than as inhibitions, the entire results appear to be devoid of any apparent inhibitory action.

One somewhat unexpected result has been the differential sex response. Female tissues are more responsive to either chemical androgens or estrogens than are those of the male; hence one must admit at the outset that zygotic sex-differentiating factors have laid the basis for differential responses by homologous structures. Thus, Müllerian ducts in the two sexes have different fundamental capacities to respond to treatment. It is to be remembered that both androgens and estrogens have been applied continuously (daily) since a stage in development when the duct was in progress of formation and continuing its growth toward the urogenital-sinus connections. At all ages of sacrifice the Müllerian ducts in the male failed to be maintained at either the ostial or the urogenital-sinus end, and the intermediate portions that remained responded weakly to such treatments as those which induce enormous responses of the same structure in the female. Since pairs of embryos in the same pouch receive identical treatment, sex is the only differential; hence, it is readily apparent that the growth and development of the Müllerian duct is by no means entirely controlled by the chemical androgens applied.

Essentially similar conditions hold for Wolffian ducts, since these structures in the female are somewhat more responsive than those of the male; in fact, Wolffian-duct response in males to androgens is remarkably unimpressive, and in the final state, though admittedly somewhat atypical in growth, Wolffian ducts in females usually exhibit much greater response to androgenic treatments than do those in males. The same is even more apparent when estradiol is administered; much larger Wolffian duct remnants are found in females than in males.

2. The question whether introduced chemical hormones override zygotic sex-differentiating tendencies may be answered by stating that, since inhibitory tendencies are not apparent, the changes revealed are those of addition of structures to those usually present. Thus, androgens applied to males cause little, if any, modification, except the intensification of development in the prostate gland and external genitalia; in females these substances induce prostate development, increase the size, and change the form of the phallus and favor retention of Wolffian ducts (normally degenerate). With regard to estrogens, the zygotic differentiating tendencies in females are tremendously supplemented, and Wolffian ducts are added in whole or in part. In males there is also a tendency to intensify development of the ducts by estrogenic treatment, since in general the Wolffian ducts exhibit a greater luminal diameter than in a similar-aged normal male; derivatives of the Müllerian duct (usually degenerate) have been maintained and may exhibit considerable response; this response is decidedly less marked in males than in females. It is clear that, in the main, zygotic sex-differentiating factors remain in ascendancy; but some redirection, or intensification, of differentiation may appear. Inhibition of heterologous characters by chemical hormones is not apparent.

3. The question of specificity of response to homologous hormones brings forth rather unusual comments, since in the majority of reports in the literature, aside perhaps from some cases in mammals, homologous responses are emphasized.

It has been repeatedly pointed out above that both sets of embryonic sex ducts show responses to treatments with either androgens or estrogens. In females an outstanding result has been the responses of Müllerian ducts to androgens (see, e.g., Pl. IV, Fig. 6, and Pl. V, Fig. 8). Whereas this female duct in males shows some responses to androgens by its persistence over a considerable proportion of its natural course, we see that in pairs of male-female such as 101A and 101B, which have received treatments continuously from a period in which formation is just under way, the Müllerian duct in males shows a

maximal luminal diameter of 0.01 mm., whereas in the corresponding female this may be more than one hundred times as great. Furthermore, Wolffian ducts, which we might anticipate would respond, do so only slightly in males and with somewhat greater intensity in females. Similarly, with regard to specificity, estrogenic treatments of females are frequently followed by Wolffian ducts as large as or larger than Müllerian ducts. Again, response of Wolffian ducts (unexpected) is greater in females than in males. It is evident, therefore, that specificity in response to chemical hormones does not characterize the developing embryonic sex ducts in the opossum.

4. The question of hormone antagonism in development has been covered in part already. It may be restated that chemical androgens do not suppress or inhibit differentiation of the female reproductive system but rather speed up and intensify development and exhibit precocious differentiation in the Müllerian system. In a similar manner estrogens have not shown antagonistic influences in developing males, with the possible exception of the prostate and phallus regions, where the type of differentiation is changed—they serve merely to add certain female rudiments that are not normally retained. Rather than inhibiting heterologous characters, these chemical hormones proved to exert a stimulating influence on opposite sex characters.

5. Before making direct comparison between the results of chemical-hormone treatment of opossums and conditions in freemartins, it is perhaps justified to review some points in the original interpretations of the freemartin condition in the light of twenty-three years' additional experience. The facts portrayed by Lillie have never been questioned, and from 1917 to the present his suggestive interpretations have withstood the rigors of numerous experimentations conducted over the world. It is to be recalled that several necessary postulates had to be proposed before the theory of hormone actions could be logically developed. At least a few of these may be mentioned.

a) The hormone theory of embryonic duct differentiation presumes that the differentiating testicle or ovary secretes a hormone that guides differentiation of the corresponding reproductive system, although Lillie mentions the possibility that the circulating substances from the male—effectively inducing modifications in the female—might have a different origin than the testis. It appears that substantial evidence is still lacking that a gonad secretes effective morphogenetic substances, or sex hormones, during the course of its early differentiation, when these would have to act to guide development of the duct system. No one has yet obtained an embryonic hormone. And, although Womack and Koch (1930) obtained comb-growth-stimulating substances from extracts of testes from calves in late fetal stages, such active material was not obtained from extracts of urine from postnatal calves. It is true that for amphibia it is generally accepted that an inductive substance (medullarin or cortexin, of Witschi) does issue from the developing gonad, but this is believed to be not a circulating hormone but something different from hormones secreted by adults. Thus, in comparing effects in females treated with chemical hormones with effects in freemartins, one must keep in mind the possibility that these effects are produced by entirely different active substances. Despite the inclinations of Burns (1938*b*) to consider it possible that the same substance is active throughout an entire ontogeny, the evidence from these investigations on opossums gives indications of a different nature.

b) Steinach, prior to 1917, had claimed a mutual antagonism between sex hormones of the nature that the presence of one hormone inhibited development of the opposite sex characters, and Lillie's demonstration of an inhibition of the ovarian cortex and Müller-

ian ducts in freemartins by substances transferred in the blood of the male would appear to lend credence both to the antagonism hypothesis advanced by Steinach and to the idea that the freemartin effects were due to a sex-hormone action. More recent studies have failed to substantiate Steinach's conceptions of hormone actions, however, and injections of chemical hormones have shown but slight inhibiting effects on sexual differentiation in the heterologous sex.

c) The explanation of the freemartin condition on the basis of the hormone theory must presume a sex specificity in response of homologous tissues to the hormone involved. The results noted on the opossum and on embryos of other species have failed to indicate such specificity of response to pure chemical hormones.

d) Modifications in the freemartin failed to involve the external genitalia, since these remained entirely female even in those specimens showing the highest grades of internal modification; hence, phallic development appeared to be the least liable to modification. Chemical-hormone application, however, has revealed the developing external genitalia to be very sensitive to modifying influences—a point stressed also in the work of Burns (1939*d*, 1939*e*).

e) Lillie recognized the problem of accounting for the protection of a developing male embryo from modifying influences of maternal hormones and suggested placental impermeability to maternal hormones as a possible protective mechanism, although other possibilities were noted. It has been shown by later observations that amniotic fluid in some species is a rich source of estrogenic substances, and normal male development is not influenced by their presence.

With these points in mind one may suggest rather directly the observations on agreement and disagreement between conditions resulting from chemical-hormone treatment of the opossum and the freemartin conditions in which, theoretically, a male hormone secreted by the embryonic testis was supplied to the female. The comparison involves only chemical androgens in females, since theoretically no estrogenic substance was present in the freemartin, owing to early suppression of ovarian differentiation; hence, the male showed no effects from ovarian secretions.

Organ	Freemartin	Female Opossum
Ovary	Cortical inhibition	No apparent effect
Müllerian duct	Inhibition	Stimulation
Wolffian duct	Retention and stimulation	Incomplete retention, mild stimulation
Prostate	Stimulation	Induction and stimulation
External genitalia	No effect	Marked stimulation in male direction

A consideration of such a diverse set of responses in the cases of these 2 females of different species to male hormones suggests such interpretations as the following: (1) species reactions are markedly different in opossum and cattle sex differentiation, (2) supply of effective substance to the modifiable system is of distinctly different order, or (3) entirely different substances are concerned in producing the observed effects. It will appear evident, with the above points in mind, that freemartin effects have not been produced in the opossum by treatments of developing young with chemical sex hormones.

6. A consideration of the influences of chemical hormones on sexual differentiation in

the young of opossums provides little or no suggestion that such substances play any role in normal sex differentiation. Prevailing ideas relative to hormonal control of sexual differentiation imply, in general, a high degree of similarity in capacity for homologous structures in the two sexes to respond with homologous stimulation and heterologous inhibition.

Experiments reported here have amply demonstrated a decided difference in the capacity of homologous ducts to respond to a given treatment, especially in the case of Müllerian ducts. Likewise, the lack of specificity in response on the part of the sex ducts during developmental stages would seem to preclude the effective operation of these hormones or other estrogenic or androgenic substances having a similar effect. Thus, amounts of androgens in males that stimulate Wolffian ducts cause retention and stimulation of residual portions of Müllerian ducts in whole or part. Whereas it is probably true that a greater dosage has been experimentally administered than would be present under normal conditions, one may presume that the suggested heterologous inhibition would be even greater than in normal development. The reverse, however, is true.

The inability to see suggestions from these experiments that would justify the idea of a hormonal control on duct differentiation, or to see that the results lend credence to the freemartin interpretations involving gonadal secreted hormones, have led to an attempt to suggest an interpretation that might plausibly cover both phases of development. Prior to making the suggestions, it appears to be advisable to review very generally the effects that have been noted from treatments of other species during developmental stages with such chemical hormones as have been employed.

Among amphibia (Burns, 1938, 1939; Ackart and Leavy, 1939; Foote and Witschi, 1939; Foote, 1940; Puckett, 1940) specificity of response has largely followed when chemical-hormone treatment has been carried out during early developmental stages, both as regards ducts and, to some extent, gonads. Where mention is made of effects on duct development, Wolffian ducts but not Müllerian have responded to androgens; and Müllerian, but not Wolffian, ducts have responded to estrogens. Burns (1939*a*) has mentioned inhibitory effects on Müllerian ducts by testosterone. Gonadal development has usually been modified in a specific sense in that androgens have favored testicular development in genetic ovaries but have been largely without effect on genetic testes, and estrogens have led to the development of genetic testes into ovaries but have shown no appreciable effects on genetic ovaries. In slowly metamorphosing forms (*Rana catesbiana*) Puckett (1940) found gonadotropic agents a necessary aid to the reactions before the sex hormones could exert their effects, since androgens or estrogens alone failed to effect gonadal modifications.

Among reptiles Risley (1937) found evidences in turtles of androgenic stimulation of ovarian medulla without inhibition of cortex, but duct responses were not emphasized. Sexually differentiated, but immature, alligators did not react so specifically to sex hormones. Androgens had no effect on Wolffian ducts of either sex, but Müllerian ducts in females were markedly stimulated. Estrogens stimulated markedly the Müllerian ducts of females and, to a lesser extent, residual portions of this structure in males but exerted no effect on Wolffian ducts. Gonads exhibited responses to estrogens by hypertrophy of ovarian cortex and any residual cortex on testes but was without effect on ovarian medulla or upon the testis proper (Forbes).

The bird exhibits a variety of responses when treated with androgens and estrogens during sexual differentiation, on account of, first, an inherent asymmetry and, second,

differential responses to androgens of slightly different chemical constitution (Dantchakoff; Kozelka and Gallagher; Wolfe and Ginglinger; Willier, Gallagher, Rawles, and Koch; Willier). In most cases a high grade of specificity was exhibited in duct responses —Wolffian ducts responding to androgens and Müllerian ducts to estrogens; but Müllerian ducts of females are not inhibited by testosterone—although they do show such a response to androsterone—and Wolffian ducts are not inhibited in males by estrogens. Exceptions to this general statement are registered by Willier, Rawles, and Koch (1938), who found that Müllerian ducts in males show persistence and stimulation after treatments with androsterone and dehydroandrosterone, whereas in females they show no response.

Gonadal responses in the bird to sex-hormone application have been decidedly variable, and for detailed consideration the reader is referred to the account of Willier (1939). In brief, testosterone exerts no appreciable effects on testes or ovary, but androsterone stimulates incipient cortex on the left testis and may change the internal portion to a typical ovarian medulla (ovotestis); the right testis may likewise be modified by a large dosage of androsterone into an ovarian medulla (cortex absent). Neither of these two chemical androgens appreciably affects the developing genetic ovary. Estrogens, while showing practically no modifying effect on genetic ovaries, exert an effect on testes similar to androsterone (left, ovotestis; right, ovarian medulla).

Among mammals, hormonal treatment during sexual differentiation has been employed on rodents (*guinea pig:* Dantchakoff; *rat:* Greene, Burrill, and Ivy; Hamilton and Gardner; Hamilton and Wolfe; Moore; *mouse:* Raynaud; Turner) and on the opossum (Burns; Moore). Since no pronounced qualitative modifications of gonads have followed either treatments with androgens or estrogens in any of these species, our attention may be limited to the question of specificity of reaction on the part of sex ducts. Generally speaking, androgenic treatment has revealed strikingly little effect on Wolffian-duct differentiation in males, but in females the duct has shown a very consistent persistence; and such outgrowths as seminal vesicles have been found in female mice and rats. Expected inhibitory effects of androgens on Müllerian ducts have not been apparent, but instead these ducts have been retained in all treated female rodents and in the male opossum; and they have been tremendously stimulated in developing female opossums. In rats and mice the distal vagina has been consistently absent after androgenic treatments, suggesting a specific inhibitory effect, but this urogenital-sinus derivative is inhibited to a similar degree occasionally in untreated young (Plagge and Lamar, 1939) and also by estrogenic treatments (Greene, Burrill, and Ivy, 1939*b*). Because of these facts it would appear justified to consider the absence of terminal vagina as a nonspecific retardation of normal developmental processes that could conceivably be brought on by many different agents rather than by a specific hormonal inhibition of heterologous characters.

Estrogenic treatment during sexual differentiation has been carried out on the rat by Greene, Burrill, and Ivy (1939*b*) and on the opossum by Burns (1939*c*, 1939*e*) and Moore (1939). Such treatments of females would be expected, on the basis of the theory of hormonal differentiation of sex ducts, to show only possible stimulation of the Müllerian system; but in the rat it was noted by Greene and associates that complete retention of Wolffian ducts provided as complete a male system of excurrent passages as were present in normal males; seminal vesicles were not noted. In the opossum, likewise, the Wolffian ducts of females were stimulated by estrogens rather than inhibited.

When one thus contemplates such an array of results as have emerged from treatments of animals undergoing sexual differentiation with androgens and estrogens, it becomes a real difficulty to accept in principle the contention that specific hormonal substances issuing from developing gonads are the controlling agents in differentiation of the reproductive system. The postulated stimulation of homologous characters and inhibition of heterologous characters does not hold.

In an attempt at a reorientation to this complex problem, four important aspects have been taken into consideration.

1. One of the more impressive aspects of this study has been the different capacity of the same embryonic structure in the two sexes to respond to the same treatment. It is to be remembered that treatments were initiated during the development of these structures and not subsequent to their differentiation. It is apparent that a Müllerian duct in a female and one in a male have strikingly different capacities to react to the same treatment. When estrogens are applied alike to females and males in the same pouch, female Müllerian ducts respond with a tremendous development in comparison with those in males. Similarly, the application of androgens to both sexes emphasizes the capacity of female Müllerian ducts for a greater response than is evidenced in male Müllerian ducts. The Wolffian duct, though showing less spectacular responses especially to androgens, proves to have different capacities to respond to those hormones despite the fact that these ducts have functioned equally in both sexes as a urinary outlet from the mesonephros during a considerable portion of the treatment. There is thus to be emphasized a zygotic differential in capacity of basic embryonic tissue for response. Burns (1939*d*, 1939*e*) has likewise stressed different capacities of the same structure in the two sexes to respond to a given treatment.

2. Our current viewpoint permits the assumption that primary sex-differentiating factors, the zygotic sex differentials, are capable of converting the undifferentiated gonad into an ovary or testis, as is proper on account of the original determination. There does not appear to be any known fact against the conception that duct differentiation or other structures (prostate gland, Cowper's gland, phallus, etc.) may likewise depend upon the same forces that molded the proper gonad development, and that duct development is parallel to gonad development, perhaps dependent upon the same differentiating factors rather than being conditioned to develop by hormones produced by these developing gonads.

3. The fact is well established that the sex ducts are capable of showing response to chemical hormones even during the course of their development. However, this need not necessarily be interpreted as evidence that their normal development depends upon such hormones. There is no longer doubt that their fundamental functional differentiation approaching puberty, and in subsequent periods, depends upon gonad-hormone secretion; but the point at issue is the period in ontogeny at which this gonadal control takes hold. Since residual tissues respond to hormones, and in these experiments give no evidence of heterologous inhibition, it is difficult to conceive how one set of ducts could survive and the other disappear if their development depended upon a sex hormone of the nature employed in these experiments; each set responds to either androgen or estrogen administration.

4. It is yet unknown at what period in gonad development hormones are first secreted; our methods are scarcely adequate to make this determination. One fact may be suggestive: gonads that are actively secreting hormones, and even prior to this demonstrable

activity, have shown, in practically all instances, a response to gonadotropic substances. If the assumption is valid that gonads capable of secreting hormones will respond to gonadotropic substances—and all previous experiments indicate that equine gonadotropin is highly similar to pituitary materials in its action—it should follow that, since gonadal response in opossums has been negative in early stages and very weak or absent even up to the 100-day period, the gonads during the stage of duct development are not secreting hormones.

With such considerations in mind, the following relatively simple working hypothesis of sexual differentiation in the opossum may be formulated.

Sexual differentiation involving gonads, as well as ducts, phallus, etc., are under the control of genetic sex-determining factors and not controlled by gonad-secreted hormones. It is possible that, in addition to the genetic factors in the localized anlage of reproductive organs, certain stimulating humoral substances issue from the entire somatic complex of the organism; and that these, rather than specific secretions elaborated by the developing gonad, favor homologous duct development. It may follow from this —and in keeping with the freemartin it would be expected—that the genic constitution of the tissue or organ in one sex could conceivably be overridden by such somatic humoral substances from the opposite sex and differentiation be redirected. Thus, on this basis, the undoubted humoral transmission of substances from the male twin to the freemartin would merely suggest that the unknown effective substances issue from the entire somatic complex of the male rather than from its developing testes. Therefore, there is postulated not only an effective substance different from sex hormones of later life but a substance that is produced in the organism as a whole rather than in the gonads. This does not deny an activity to gonad-secreted hormones on sexual equipment after its differentiation, but it does suggest that such hormones, later certainly effective, probably have no place in controlling early differentiation of the essential equipment of the system.

Experiments designed to test the necessary corollaries of such a conception are under way but are thus far too incomplete to warrant discussion. A tendency to conceive of a mechanism involved in sexual differentiation different from that generally current, sometimes designated as the "dihormonic theory," is already evident in Weisner's (1935) development of a "monhormonic" theory. In contrast, the suggestions here offered might be labeled "anhormonic"; but it is considered premature to make a commitment with regard to hormonal substances. It appears probable that humoral substances do play a part in giving expression to inherent potencies carried by the genetic constitution of the tissues; and these substances, if real, may ultimately be considered to be hormones. But the evidence suggests that, whereas in the later physiology of the reproductive system gonad-secreted hormones probably play the leading conditioning role, one should not deny the possible operation of a different humoral mechanism, provided, perhaps by substances produced by the whole embryo instead of by the developing gonads.

In point of time, the limits of operation of the nongonadal and gonadal humoral substances are ill defined and must await a determination of the time of appearance of effective gonadal secretions. That the nongonadal humoral factors may operate well after birth and may be decidedly overlapped by the gonadal secreted substances appears to be indicated by the work of Price (1936, 1939, 1941) on the prostate gland of the rat. Since remarkable species differences exist and since any biological reaction is dependent upon the capabilities of tissue responses as well as upon the inciting agent, one must ex-

pect differences in time, degree, and capacity of response. Relative to the basic free-martin responses, Lillie's portrayal of which has provided the stimulus for so much commendable effort in this field in the last 23 years, it should be pointed out that Wislocki (1939) has revealed a condition in marmoset twinning that should provide a duplication of the condition occurring in cattle without the appearance of any suggestion of similar responses.

VIII. SUMMARY AND CONCLUSIONS

A. 1. The newborn opossum is sexually undifferentiated and possesses a functional mesonephros for approximately 2 weeks.
   2. Pouch young become fixed on the nipples and retain an uninterrupted attachment for 60–70 days.
   3. Forty stages of normal reproductive-system development, involving both sexes, have been studied in serial section to include the period between days 1 and 100.
   4. Gonad differentiation suggests ovaries or testes by the third day.
   5. Müllerian ducts appear by day 3 and reach the urogenital sinus at approximately day 10. In females further development produces by day 30 a definite regional differentiation into oviducts, uterus, cervix, and lateral vaginal canals. In males these ducts atrophy progressively in a posteroanterior direction subsequent to day 10. Small anterior rudiments remain along the epididymis and exhibit increased growth from about day 80; by day 100 they are short, luminate tubes.
   6. Wolffian ducts exhibit retrogression on termination of urinary function in both sexes. In females the ducts atrophy progressively in a posteroanterior direction; epoöphoron tubules are conspicuous.
   7. The prostate gland arises in the male as epithelial buds from the urogenital-sinus epithelium along its course on day 16; in females, the prostate anlage does not appear at any stage of development. Prostate buds appear in males by day 15; branchings of original outgrowths occur about day 40; by day 100 the branching cords permeate the mesenchyme of the urogenital-sinus wall and constitute the foundations for future glands; by the 8th month central ends of the glands are canalized; and the adult type of inclosed prostate is present at 1 year of age.
   8. Testes show little spermatogenetic activity up to the 100th day; by 1 year spermatozoa are being produced. Ovaries at the 100th day contain numerous follicles with large ovocytes, but no antra.

B. Applications of androgenic substances were made by ointment, or by injection, in both sexes, covering periods from day 3 to day 100 and in a few older individuals. Serial sections of the reproductive system were prepared of 18 males and 22 females treated with androgens.
   1. Androgens exert only mild stimulation on Wolffian ducts in males and a somewhat greater effect on female Wolffian ducts; these latter are preserved in females in an atypical manner.
   2. On Müllerian ducts mild effects are visible in males, extreme stimulation occurring in females. Precocious development of the female tract involves growth, regional differentiation, and glandular developments. Cessation of treatment does not readily involve regression.
   3. Prostatic development in males is markedly hastened. In females, induction of prostate formation occurs, as well as precocious stimulation of growth and differentiation.

4. Testes and ovaries are apparently little affected by androgenic treatment.
5. Phallic stimulation is pronounced in both sexes; precocious differentiation is readily induced. The female phallus is converted into a typical penis with bifurcate glans.

C. Application of estrogenic substances to both sexes involved skin ointments and subcutaneous injections. Estrogenic substances are decidedly toxic and caused the death of many treated young. Serial sections of the reproductive system were prepared of 8 treated males and 11 estrogen-treated females.
   1. Wolffian ducts in both sexes have been stimulated, but more appreciably in females than in males.
   2. Müllerian ducts in males are not maintained in normal relations, but localized remnants become large luminate segments. In females the ducts are precociously stimulated, and glandular differentiation greatly hastened.
   3. The urogenital sinus is especially sensitive to estrogenic substances; the epithelial lining, in both sexes, exhibits tremendous hyperplasia and metaplasia, and cornified cells are sloughed off into the lumen.
   4. Prostate development in males does not occur, for the resulting cell migration is directed centrally rather than peripherally; previously formed prostate outgrowths are converted into atypical hyperplastic and metaplastic growths of enormous proportions.
   5. Ovaries or testes are not appreciably modified in their development.
   6. External genitalia are increased in size by estrogenic treatments, but the type of growth is decidedly different from that following androgenic substances; it is an increase in all directions, resulting in a rounded projecting prominence rather than the typical elongate penis type.

D. Zygotically different responses have been noted from the fact that—
   1. Wolffian-duct stimulation by androgens is relatively greater in females than in males;
   2. Müllerian ducts in males show comparatively little response to androgens, whereas in females tremendous precocious stimulation results from treatment;
   3. Wolffian ducts in males respond less markedly to oestrogenic substance than do those in females;
   4. Müllerian ducts are not retained in their normal relations in males, whereas in females precocious differentiation occurs.

E. Quite similar responses follow in each sex in (*a*) androgenic effects on the prostate, though induction occurs in females and (*b*) on external genitalia; and in (*c*) estrogenic effects on the urogenital sinus and on external genitalia.

F. Gonadotropic agents evoke little or no response at early ages, and further work is required to demonstrate the capacity of gonads to secrete hormone in the early stages of their development.

G. Reasons are given for suggesting that early duct differentiation is not dependent on gonad-secreted hormones, and a tentative working hypothesis is suggested.

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PLATE I

FIG. 1.—Photograph of open marsupial pouch showing young on day 3 attached to nipples (*see low center*). About $1\frac{1}{2}\times$.

FIG. 2.—Opossum pouch young on day 35. Note nipple attachment and vacated nipple from which a treated young had just been removed. Note prominence of external genitalia from androgenic treatments. About $1\frac{1}{2}\times$.

PLATE II

FIG. 3.—Drawing of normal adult male reproductive system; tunica removed from testis on right.

PLATE III

FIG. 4.—Drawing of adult normal female reproductive system.

PLATE IV

FIG. 5.—Drawing of 31-day normal female reproductive tract (ser. 18). *A*, adrenal; *Bl*, bladder; *K*, kidney; *LVC*, lateral vaginal canal; *OV*, ovary; *Ovd*, oviduct; *Ut*, uterus.

FIG. 6.—Drawing of 31-day treated female reproductive tract (ser. 64B). Testosterone-propionate treatments on days 10–30. Drawn to scale of Fig. 5. *A*, adrenal; *Bl*, bladder; *K*, kidney; *LVC*, lateral vaginal canal; *Ut*, uterus; *WD*, Wolffian duct.

PLATE V

FIGS. 7–16.—Photomicrographs from opossum serial sections (same magnification for all figures). Original, 30×.

C, cervix
LVC, lateral vaginal canal
MD, Müllerian duct
O, ostium
OV, ovary
UGS, urogenital sinus
UT, cavity of uterus
UTG, uterine glands
WD, Wolffian duct

FIG. 7.—Normal female, 31 days old (ser. 18; see Fig. 5), showing cervical canal in genital cord and sections through uterus. Gut above.

FIG. 8.—Androgen-treated female, 31 days old (64B; see Fig. 6), showing enormous development of the Müllerian duct into a uterine cavity, precociously differentiated uterine glands, ostium, and ovary; Wolffian ducts are visible.

FIG. 9.—Androgen-treated male, 31 days old (64A; same treatment as female in Fig. 8). Two sections of the Wolffian duct reveal its relative unresponsiveness; Müllerian duct, though preserved, exhibits minimal response (cf. Fig. 8). Gut above; urogenital sinus (*center*) shows precociously developed prostate (cf. Fig. 10).

FIG. 10.—Normal male, 32 days old (34B), showing early prostate-cord outgrowth from urogenital-sinus epithelium.

FIG. 11.—Androgen-treated male, 30 days old (101A). Prostatic outgrowths from urogenital-sinus epithelium greatly exceed the normal (cf. Fig. 10).

FIG. 12.—Androgen treated female, 30 days old (101B). Prostatic induction and precocious development (compare with normal male, Fig. 10).

FIG. 13.—Estrogen-treated male, 31 days old (39A). Urogenital-sinus epithelium shows tremendous hyperplasia and metaplasia; proliferation, instead of peripheral for prostate formation, has been central with sloughing of cells into the lumen of the urogenital sinus.

PLATE I

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PLATE II

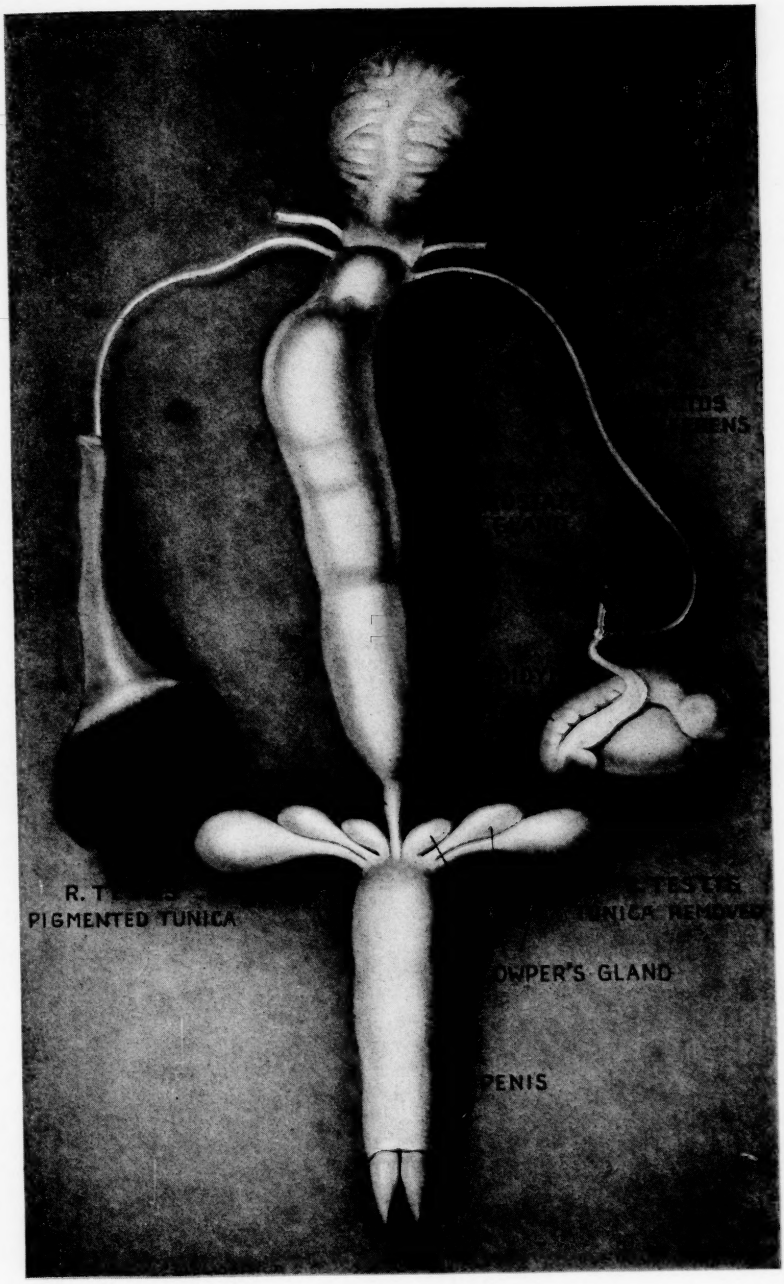

FIG. 3

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PLATE III

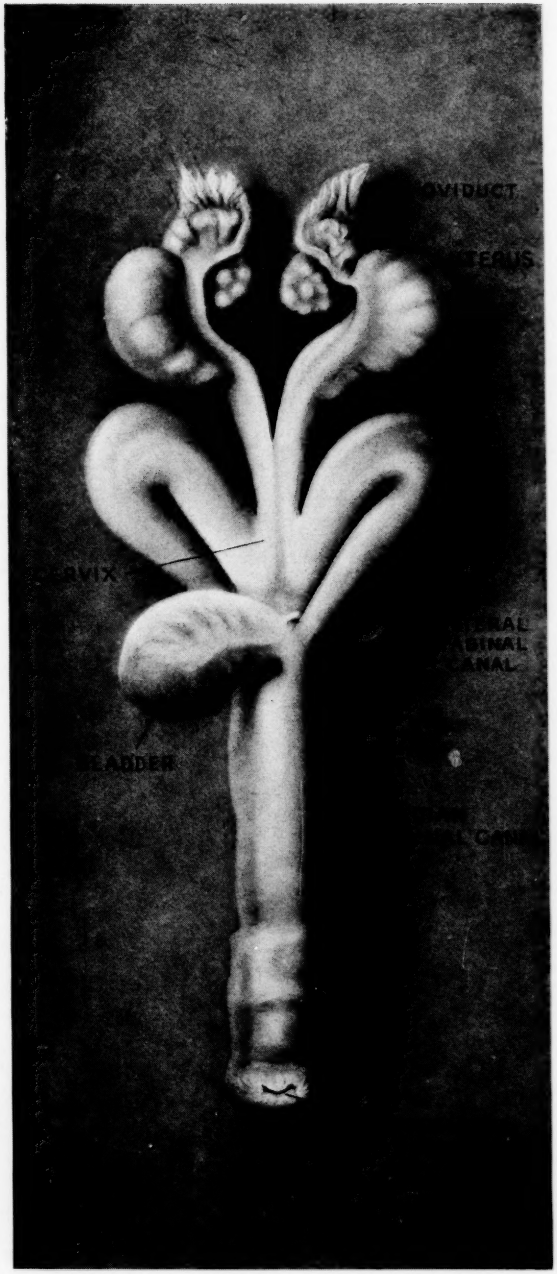

FIG. 4

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PLATE IV

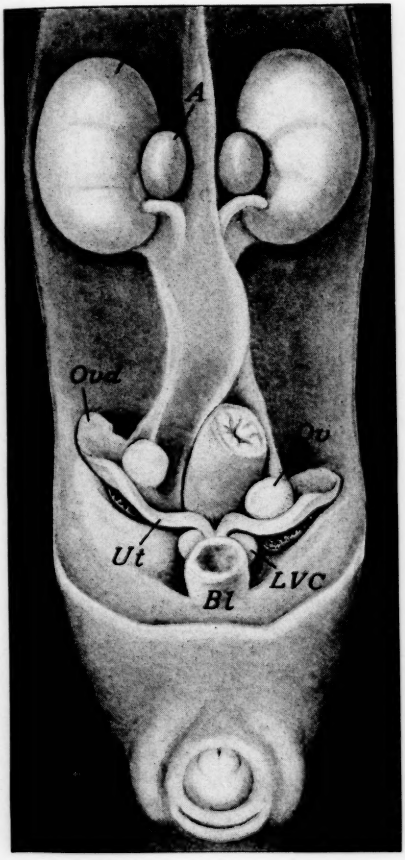

FIG. 5

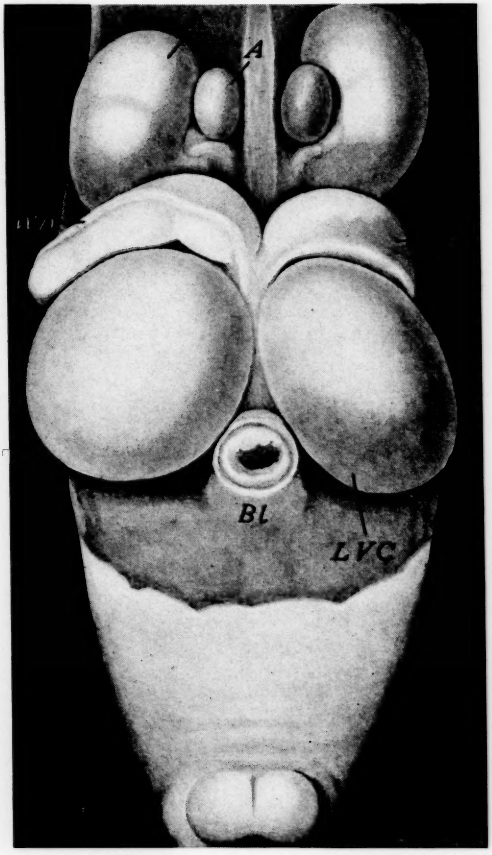

FIG. 6

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PLATE V

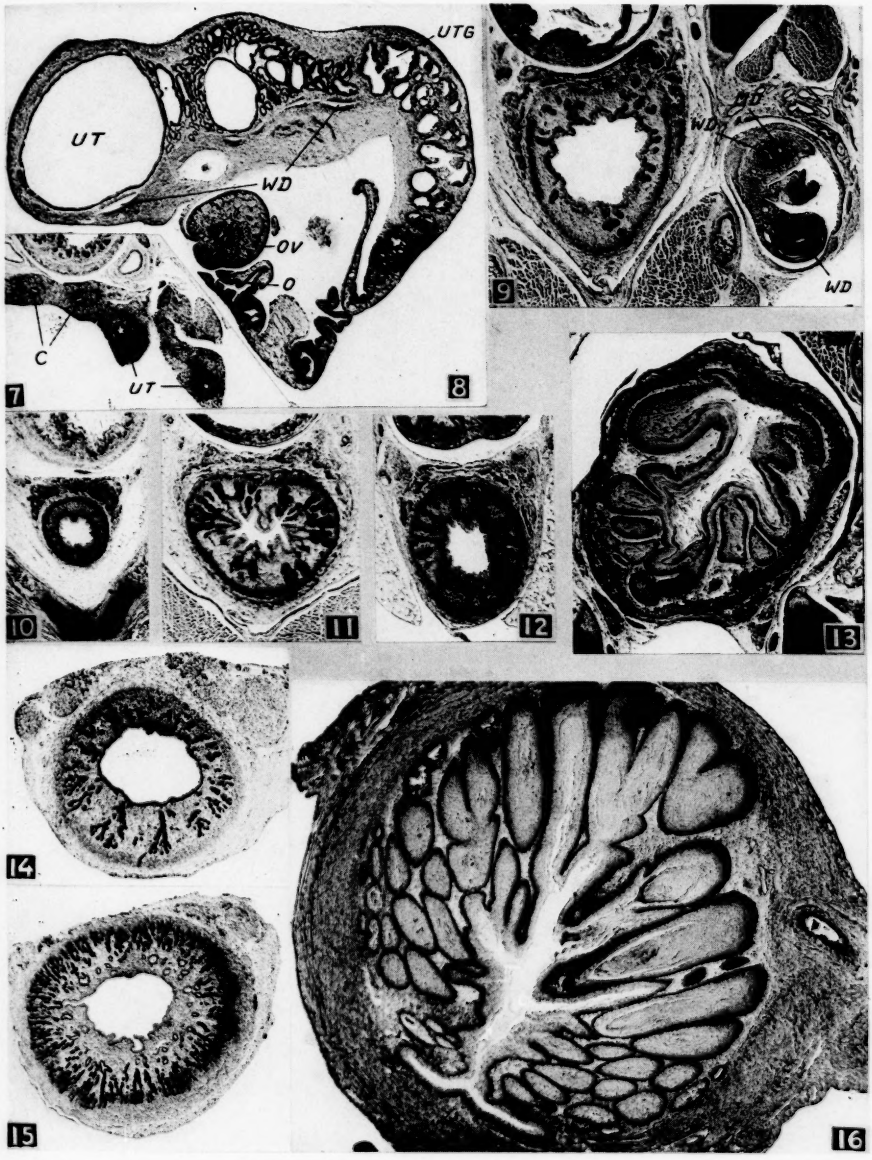

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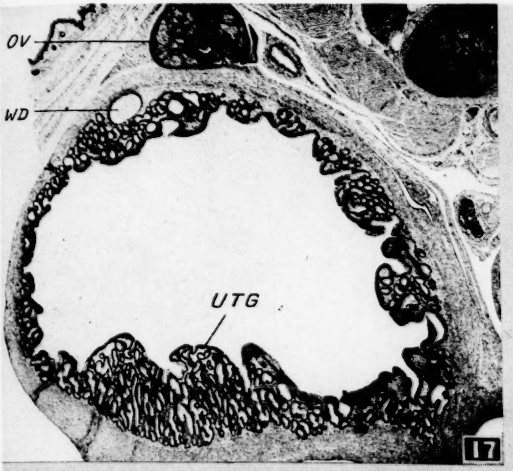
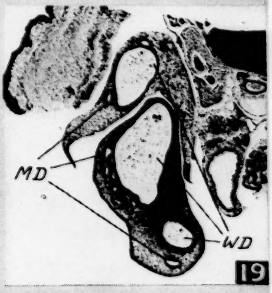
PLATE VI





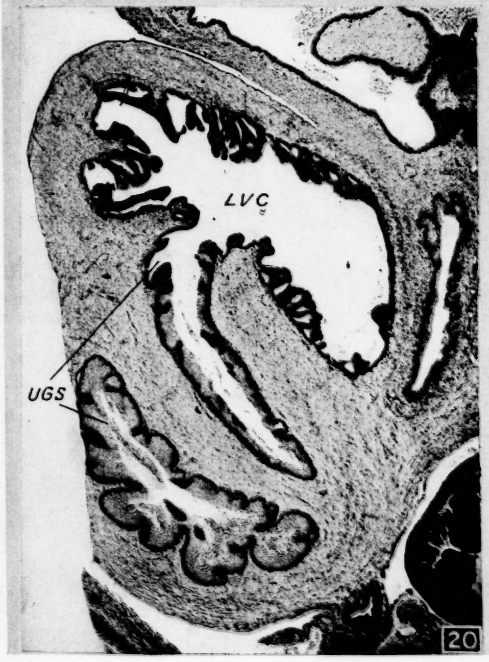


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FIG. 14.—Untreated male, 100 days old (88), showing prostate gland as solid cords of cells, without canalization.

FIG. 15.—Androgen-treated female, 95 days old (118); treatments on days 3–44; no treatment on days 45–95. Prostate-gland induction, and differentiation much beyond normal male of age (cf. Fig. 14.).

FIG. 16.—Estrogen-treated male, 83 days old (93); treatments on days 70–82. The previously formed prostatic outgrowths and urogenital-sinus epithelium have undergone tremendous hyperplasia and metaplasia.

PLATE VI

FIGS. 17–22.—Photomicrographs from opossum serial sections (magnifications same in Pls. V and VI). Original, 30×. (For labels see Pl. V.)

FIG. 17.—Estrogen-treated female, 46 days old (ser. 115). For increased uterine diameter compare with Fig. 7; for precocious uterine-gland development compare with 100-day normal female (Fig. 22). Ovary appears normal. Wolffian duct slightly posterior becomes size of uterus in this section.

FIG. 18.—Estrogen-treated male, 23 days old (55A).

FIG. 19.—Estrogen-treated female, 23 days old (55B). For greater Wolffian-duct response to estrogens than to androgens compare Figs. 18 and 19 with the androgen-treated male of Fig. 9.

FIG. 20.—Estrogen-treated female, 46 days old (115; same young as Fig. 17), showing transition of glandular epithelium of lateral vaginal canal into upper portion of urogenital sinus.

FIG. 21.—Androgen-treated female, 95 days old (117). This androgen-stimulated uterus, with its glandular differentiation, should be compared with the normal 100-day uterus of Fig. 22.

FIG. 22.—Normal female 100 days old, to show cross-section of the uterus; no glandular differentiation.

AN EXPERIMENTAL STUDY ON THE ORIGIN OF MELANOPHORES IN BIRDS

(Four figures and three plates)

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THE origin of pigmentation in the integumentary structures of vertebrates has been widely discussed from the time of the earliest investigations. The first authors were impressed by the occurrence in the epidermis and dermis of very characteristic starlike cells carrying melanin granules. Two questions arose: Do these cells belong to the mesoderm or to the ectoderm? Is the melanin pigment formed only in these cells, or do other cells have the capacity to produce it? The answers which have been given to these questions up to the present time can be classified into three groups. As early as 1860 Kölliker described these pigment cells in *Protopterus* and concluded that they were derived from mesenchymal cells which migrated into the epidermis. This view was confirmed by Kerbert (1877) for reptiles and birds, Riehl (1884) for man and birds, and Ehrmann (1885) for man, various mammals, and birds. Haecker (1890) studied pigmentation in bird feathers and came to the conclusion that pigmentation of feathers was caused by migrating pigment cells of mesenchymal origin.

However, such investigators as Mertsching (1889), Klee (1886), Strong (1902), and Winkler (1910) reached quite a different interpretation. In their opinion the pigment was formed within the epidermal cells of amphibians, birds, and mammals. The pigment cells in the mesenchyme were believed to be derivatives of epithelial cells as Post (1894) had pointed out. Still other investigators (Jarisch, 1892; Caspary, 1891; Lloyd-Jones, 1915; Greite, 1934) tried to combine both viewpoints. While they recognized migrating pigment cells, they attributed to the epithelial cells also the capacity to produce melanin.

It is evident that the source of the pigment cells cannot be decided by histological methods alone, since in their early stages they cannot be distinguished from other cells. Experiments in amphibians lead to a strikingly different interpretation. Harrison (1910), Weidenreich (1912), and Holtfreter (1933) obtained evidence which showed that the melanophores of amphibians are not derived either from the epidermis or from the mesenchyme but from the neural crest. Du Shane (1934, 1935) was able to demonstrate this in *Ambystoma* by xenoplastic transplantations of neural crest and control tissues. The appearance of donor melanophores in hosts could be checked, since the different species have specific pigment cells.

In the chick the problem was attacked by Dorris (1936, 1938, 1939). From studies in tissue culture and grafting to the leg-bud stage she showed that neural crest produced melanophores, but skin and mesoderm failed to do so. Willier and Rawles (1940) have found that grafts of wing or leg skin and mesoderm to the wing bud produce melanophores only for donors beyond a certain age. Watterson (1938) obtained similar results for

[1] Research fellow in embryology. I wish to thank Professor B. H. Willier for initiating this problem and for his interest during the course of the work.

pure mesoderm. Eastlick (1938 and 1939*b*) has shown that the pigment-producing agent must originate in the neural-tube region and spread laterally in later development. Leg buds of 70-hour chick embryos of pigmented breeds, cut at the edge of the neural tube and transplanted into the coelom of White Leghorn embryos of the same age, developed pigmented down feathers. If severed at the level of the intermediate cell mass, however, unpigmented down resulted. Furthermore, in older embryos the more distal parts of the limbs, if grafted into the coelom, developed pigment.

The present investigation was designed to obtain further evidence from experimental and histological studies on the extra-epidermal origin of the melanophores in birds and their derivation from the neural crest. In addition, the origin of the retinal and chorioid pigment of the eye was analyzed.

MATERIAL AND METHODS

To test the potency of melanin formation various tissues of bird embryos of pigmented breeds and of different ages and levels were transplanted to host embryos. Small isolated pieces were grafted (1) into the wing bud of 70-hour White Leghorn embryos; (2) into the coelom of 60–70-hour White Leghorn embryos; (3) onto the chorio-allantois of White Leghorn, Barred Plymouth Rock, and F_1 hybrid (Barred Rock ♀ × Rhode Island Red ♂). The technique used for the first series of experiments was described by Willier, Rawles, and Hadorn (1937) and by Rawles (1939); for the second by Hamburger (1938); for the third by Rous and Murphy (1911), and for embryological research by Willier (1924).

The donors were usually Barred Plymouth Rock; but various pigmented breeds of fowl including Brown Leghorn, New Hampshire Red, F_1 hybrid (Barred Rock ♀ × Rhode Island Red ♂) were also used. In addition, Mongolian and Formosan Pheasants, robin (*Turdus migratorius*), and Black Fantail Pigeon served as donors.

Selected grafts were fixed in Bouin, sectioned at 7 and 12 μ, and stained with Delafield's haemotoxylin and eosin. Such preparations demonstrated clearly the absence or presence of melanin. Further details will be given in the appropriate sections.

EXPERIMENTS

I. ORIGIN OF MELANOPHORES OUTSIDE OF EPIDERMIS AND MESENCHYME

The experiments of Dorris (1936, 1938, 1939) and Eastlick (1938 and 1939*b*) mentioned above show a high degree of independence of the melanin pigmentation and epidermal structures. This is even more strikingly demonstrated by xenoplastic grafts of embryonic robin tissue into the wing bud of White Leghorn embryos (Rawles, 1939) where White Leghorn feathers carrying specific robin pigment granules were obtained.

To obtain further evidence for this independence and to trace the lateral spreading of the pigment-forming capacity, pieces of embryos of varying age were isolated and transplanted onto the chorio-allantois of 8-day hosts or into the coelom of White Leghorn embryos of 60–70 hours. Isolates of about 1 somite in length were cut at different longitudinal levels and at varying lateral distances from the neural tube. The cut, for instance, ran between the neural tube and the somite through the intermediate cell mass or through the middle of the limb bud, etc.

The chorio-allantoic grafts were examined with a dissecting microscope, and the presence or absence of melanophores ascertained. Certain grafts were sectioned and

studied for the presence of pigment cells. The hosts with coelomic grafts were killed before hatching and dissected. It was found that the transplants which developed in the

TABLE 1*

ORIGIN OF MELANOPHORES OUTSIDE OF EPIDERMIS AND MESENCHYME

Case No.	Donor	Level	Isolate	Host	Melanophore Production	Remarks
			A. Fowls			
16-12	BR 3 s.	node	node+ heart	WL ch-a.	+	
16-2	BR 3 s.	3 s.	neural fold	WL ch-a.	+	contracting heart muscles
48-13	BR 11 s.	8/9 s.	nt-som	WL coelom	−	White down
6-2	BR 18 s.	16 s.	nt-som	WL coelom	−	White down
6-3	BR 18 s.	16 s.	nt-som	WL coelom	−	White down
15-7	BR 18 s.		forebrain floor	BR ch-a.	+	
11-7	Red 19 s.	node	node	WL coelom	+	Red down
39-2	BR 23 s.	10 s.	nt-som	WL ch-a.	−	
13-3	BR 25 s.	16 s.	nt-som	WL coelom	−	White down
39-7	BR 25 s.	22 s.	nt-som	BR ch-a.	−	
22-7	BR 27 s.	16 s.	nt-som	WL ch-a.	−	
12-15	BR 29 s.	16 s.	nt-som	WL ch-a.	+	
30-16	F_1 30 s.	16 s.	nt-som	WL coelom	+	Black down on abnormal wing
35-2	BR 30 s.	19 s.	nt-som	WL ch-a.	+	
44-5	BR 30 s.	22 s.	nt-som	BR ch-a.	−	
44-11	BR 30 s.	bseg.	nt-som	BR ch-a.	−	
44-14	BR 31 s.	22 s.	imt	BR ch-a.	−	
52-4	BL 34 s.	25 s.	imt	F_1 coelom	−	F_1 pigment invaded graft†
41-4	BR 34 s.	33 s.	nt-som	BR ch-a.	−	
9-7	BR 37 s.	16 s.	imt	WL ch-a.	−	Feather germs without melanophores
9-8	BR 37 s.	16 s.	distal half s.	WL ch-a.	−	
7-2	BR 37 s.	head	skin	WL coelom	+	Pigmented skin and down
12-18	BR 47 s.	26 s.	tip leg bud	Red ch-a.	−	
9-12	BR 47 s.	26 s.	ventral skin	WL ch-a.	−	
			B. Other Birds			
55-5	RP 16 s.	10 s.	nt-som	WL coelom	+	Skin and down with melanophores
55-6	RP 16 s.	bseg.	nt-som	WL coelom	−	White down
57-5	Robin 7s.	bseg.	nt-som	BL coelom	−	Few BL melanophores invaded graft†
60-8	BPg 2 days	15 s.	nt-som	WL coelom	−	White down

* BR = Barred Plymouth Rock; WL = White Leghorn; BL = Brown Leghorn; Red = New Hampshire Red; F_1 = Hybrid (Barred Rock ♀ × Rhode Island Red ♂); RP = Ringnecked Pheasant; BPg = Black Fantail Pigeon; ch-a. = chorio-allantois; nt-som = cut between neural tube and somite or derivatives; s. = somite; bseg. = behind segmental plate; imt = cut through intermediate cell mass; + = presence of a structure; − = absence of a structure.

† The different breeds of fowl and species of birds have specific melanin granules (Danforth, 1937; Rawles, 1939).

body cavity were attached to the peritoneum of the body wall, to the mesenteries, or to both. Very often the connection was only a strand of tissue containing a blood vessel. The skin ectoderm of the implant developed in the majority of cases into a sac with the

down feathers directed inward (see Pl. I, Fig. 2). Less often the feathers grew directly into the body cavity (see Fig. 1). The results of the transplantations are given in Table 1. Comparing the different transplants, one can see that the pigment-producing agent spreads laterally from the neural tube, first in the anterior region and gradually in more posterior levels.

An analysis of these data leads to the following conclusions:

1. No pigment is formed in either epidermal or mesenchymal cells. The pigment-producing agent therefore comes from outside of the mesenchyme and skin. In very young donors only the neural-tube region gives pigment. In older embryos more and more lateral tissues can produce pigment cells. This shows that some factor necessary for pigment formation migrates laterally, first in the more anterior levels and later in the posterior parts of the embryo.

2. The source of the pigment lies in the region of the neural tube. At every age and level the neural tube gives pigment. At the wing-bud level the pigment-producing agent invades the tissues adjacent to the neural tube between the 27-somite and the 29-somite stages (e.g., cases 22-7; 12-15 in Table 1).

3. No difference in melanophore production is found in chorio-allantoic grafts on the White Leghorn or Barred Rock and F_1 hybrid host embryos. This excludes the presence of any differential substance in the blood stream necessary for pigment production.

II. DEVELOPMENT OF THE NEURAL CREST AND THE APPEARANCE OF MELANOPHORES IN GRAFTS

Du Shane has shown that melanophores are derived from the neural crest in amphibians. Considering the results of the preceding experiments, we immediately think of the neural crest as the source of the pigment cells, since neural-crest cells are known to have the capacity to migrate in the body. The problem now is to find out whether there is any correlation between the development of the neural crest and the observed spreading of the pigment-forming agent.

In 1928 Holmdahl showed in a very extensive and thorough study of the neural crest in birds (and mammals) that in the chick the neural crest arises first in the midbrain region of a 6-somite embryo. It never forms a continuous ridge throughout the embryo but develops as a wave proceeding caudally. The typical development at any level may be described as follows. The tissue of the dorsal part of the closed neural tube, covered by a thin layer of epidermis, becomes loose and irregular. Cells from this loose tissue form an irregular swelling on both dorsolateral edges of the neural tube. Later these cells leave the neural tube, migrating in strands both ventrally along the lateral wall of the neural tube and laterally between the dermatome and the skin ectoderm. These strands often form a characteristic fork at the edge of the dermatome. Are these cells migrating out from the neural crest the precursors of the melanophores?

The following experiments link the neural-crest cells with the appearance of melanophores. Chick embryos of about 20–30 somites show the neural crest in all stages of its development. In an anterior level the neural-crest cells have left the neural tube and have wandered out along its lateral walls and dorsal to the dermatome (Fig. 6). Posteriorly, we find either the neural-crest cells in a ridge on both dorsolateral edges of the neural tube (Fig. 5) or no morphological indication of a neural crest at all.

It is therefore possible to cut lateral pieces at different levels along the edge of the neural tube so that the anterior ones include neural-crest cells and the posterior ones do

not. With a sharp iridectomy knife such pieces were cut away from the neural tube on the right side of the donor. The corresponding left side was fixed and sectioned (Fig. 15).

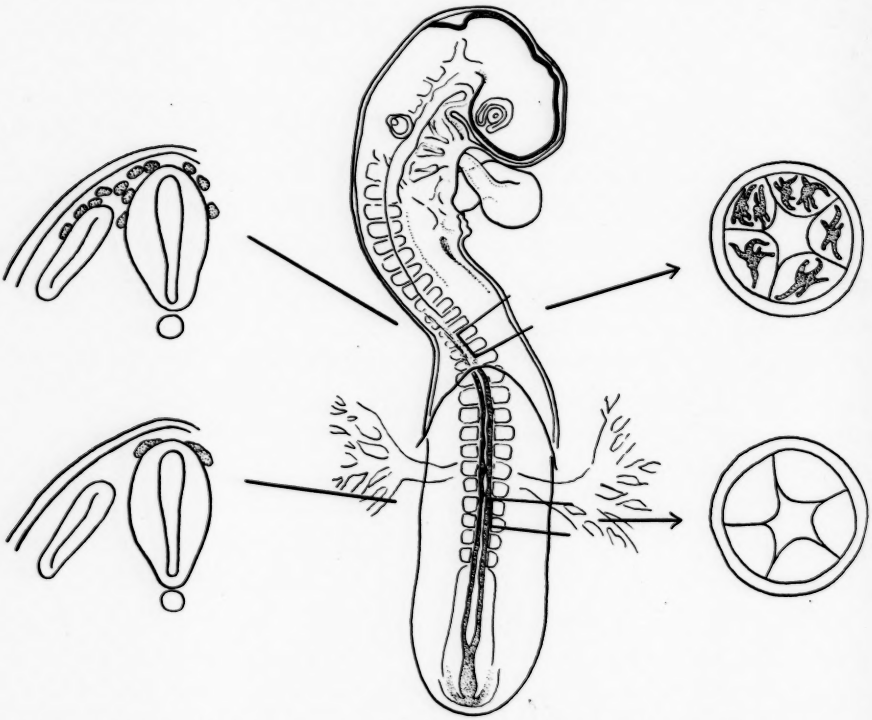

FIG. 15.—Diagram showing the correlation between the development of the neural crest and pigment formation in chorio-allantoic grafts. The right side shows the manner of isolation and whether or not melanophores are formed. On the left side the stages of neural-crest development in levels corresponding to the isolates are seen.

TABLE 2*

CORRELATION BETWEEN THE DEVELOPMENT OF THE NEURAL CREST AND THE APPEARANCE OF MELANOPHORES IN GRAFTS

Case No.	Donor	Level	Neural-Crest Stage	Host	Melanophore Production
39-2.....	BR 23 s.	10 s.	Crest	WL ch-a.	—
44-1.....	BR 30 s.	bseg.	Not formed	BR ch-a.	—
35-10.....	BR 30 s.	26 s.	Migrating cells	WL ch-a.	+
44-17.....	BR 31 s.	bseg.	Not formed	BR ch-a.	—
34-8.....	BR 31 s.	25 s.	Migrating cells	BR ch-a.	+

* For abbreviations see note to Table 1.

Thus the appearance of melanophores in the graft can be compared with the outgrowth of the neural crest. The results of these experiments are summarized in Table 2.

The data given in Table 2 show that there is a close relationship between the stage reached in the development of the neural crest and the appearance of melanophores in grafts of pieces lateral to the neural tube. In all cases where no pigment was found in the ectodermal and mesodermal structures of the graft histological examination of the intact side of the donor showed that the neural-crest cells formed a ridge dorsolateral to the neural tube. No cells had migrated from the ridges. The grafts therefore contained no crest cells (Fig. 5). On the other hand, when the cells had left the neural crest and could be seen over the dermatome (Fig. 6), the grafted piece cut at the edge of the neural tube contained neural-crest cells. In these cases the transplant on the chorio-allantois contained an abundance of melanophores. Melanophores, therefore, are produced only by grafts containing neural-crest cells.

III. THE NEURAL CREST AS THE SOLE SOURCE OF THE MELANOPHORES

Although all the evidence at hand points strongly to the neural crest as the source of the melanophores, the possibility still remains that they may arise from some other region of the neural tube. In 1939 Jones presented evidence that sheath cells—another derivative of the neural crest—may take their origin also from the ventral floor of the neural tube.

It was not found possible to isolate neural crest of the chick embryo entirely free from neural-tube and skin-ectodermal cells. Invariably, some of these cells are included. Consequently, the development of melanophores in such grafts does not prove their sole origin from the neural crest, contrary to what Dorris (1936, 1938, 1939) has maintained.

It is possible, however, to cut pieces of the neural tube (1–2 somites in length) into dorsal and ventral parts and to test their pigment-forming capacities. If such pieces are taken at an age or level at which the neural-crest cells have not yet migrated along the lateral walls of the neural tube, the ventral half would be free of them. The dorsal part contains either the presumptive or the morphologically observable neural crest (Fig. 16).

These dorsal and ventral parts of neural tube were first grafted into the wing bud of 72-hour White Leghorn embryos (Table 3, ser. 26, 27, 33). But, as the graft is generally absorbed in the wing bud, it was considered more suitable to transplant the pieces into the coelom so that a distinct graft could be obtained in all cases. A slit was made anterior to the right-leg bud of 60–70-hour White Leghorn embryos, and the implant pushed into the coelom. This site was found to provide the best conditions for growth. Table 3 gives the data of these experiments.

The grafts in the wing bud show that the ventral half of the neural tube fails to produce any pigment, if it does not contain neural-crest cells (see Table 3, cases 27-5; 27-7; 27-3; 26-8). When the neural-crest cells have migrated ventrally, the grafted ventral portion will produce melanophores (see cases 26-6; 33-4; 33-6). All dorsal grafts produced melanophores in the skin and feathers of the right wing of the host.

Still more convincing are the transplants which develop in the coelom of White Leghorn embryos. The ventral and dorsal halves of neural tube formed distinct grafts, attached either to the coelomic epithelium of the body wall adjacent to the mesonephros (Fig. 7) or to the mesenteries (Fig. 8).

The ventral half, containing no neural-crest cells, differentiates into a small body

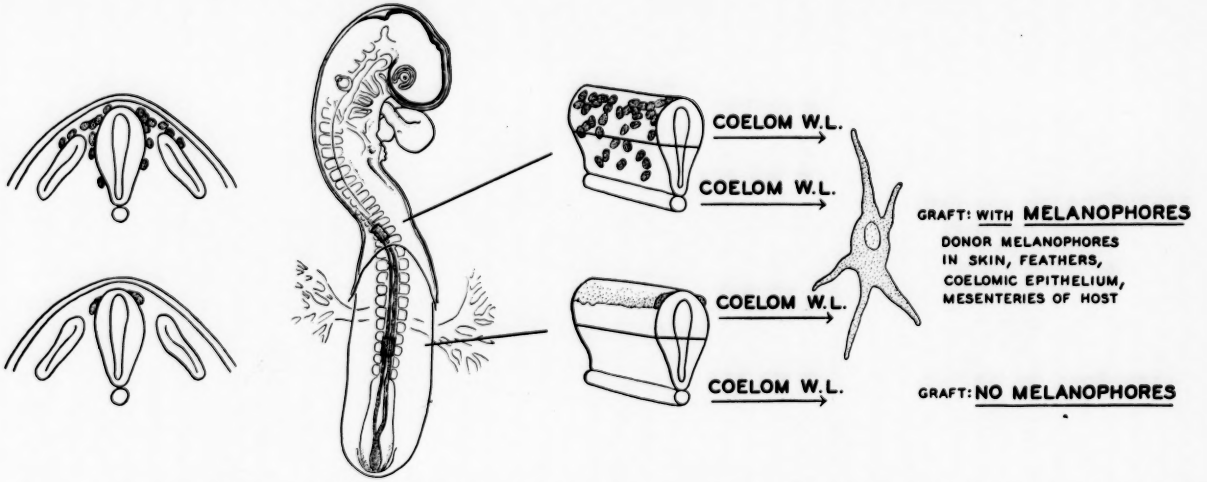

FIG. 16.—Diagram showing the isolation of dorsal and ventral halves of the neural tube at different levels of the donor embryo, together with the stage of development of the neural crest. Whether or not these isolates when transplanted to the coelom of the White Leghorn give rise to melanophores is shown at the right.

TABLE 3

THE NEURAL CREST AS SOLE SOURCE OF THE MELANOPHORES

A. FOWLS

Case No.	Donor	Level	Neural-Crest Stage*	Implant	Host	Melanophore Production
27-5	BR 9 s.	9 s.	Not formed	Ventral half†	WL wing bud	−
27-6	BR 9 s.	5 s.	Not formed	Dorsal half	WL wing bud	+
27-7				Ventral half	WL wing bud	−
26-5	BR 11 s.	5 s.	Migrating cells	Dorsal half	WL wing bud	+
26-6				Ventral half	WL wing bud	+
26-7	BR 11 s.	9 s.	Crest	Dorsal half	WL wing bud	+
26-8				Ventral half	WL wing bud	−
33-7	BR 13 s.	3 s.	Migrating cells	Dorsal half	WL coelom	+
33-8				Ventral half	WL coelom	+
47-2	BR 18 s.	17 s.	Crest	Ventral half	WL coelom	−
47-3	BR 18 s.	14 s.	Crest	Ventral half	WL coelom	−
65-5	BR 19 s.	bseg.	Not formed	Ventral half	WL coelom	−
46-4	BR 22 s.	22 s.	Crest	Ventral half	WL coelom	−
30-2	F_1 23 s.	7 s.	Migrating cells	Dorsal half	WL coelom	+
30-3				Ventral half	WL coelom	+
40-8	F_1 30 s.	11 s.	Migrating cells	Ventral half	WL coelom	+
40-4	F_1 33 s.	10 s.	Migrating cells	Ventral half	WL coelom	+
27-2	BR 35 s.	25 s.	Crest	Dorsal half	WL coelom	+
27-3				Ventral half	WL coelom	−
42-8	BR 35 s.	30 s.	Crest	Ventral half	WL coelom	−
42-9	BR 35 s.	28 s.	Crest	Dorsal half	WL coelom	+
42-10				Ventral half	WL coelom	−
33-4	BR 37 s.	35 s.	Migrating cells	Ventral half	WL wing bud	+
33-5	BR 37 s.	30 s.	Migrating cells	Dorsal half	WL wing bud	+
33-6				Ventral half	WL wing bud	+
9 grafts from dorsal halves from various levels and ages					WL wing bud and WL coelom	+

B. OTHER BIRDS

Case No.	Donor	Level	Implant	Host	Melanophore Production	Remarks
56-5	MP‡ 26 s.	24 s.	Ventral half	WL coelom	−	
56-6	MP 26 s.	20 s.	Ventral half	WL coelom	−	
56-11	FP 39 s.	.....	Neural tube	WL leg bud	+	Leg with pheasant-colored down
57-7	Robin 7 s.	bseg.	Ventral half	BL coelom	−	No robin melanophores
57-8	Robin 7 s.	7 s.	Ventral half	WL coelom	−	
57-9	Robin 7 s.	4 s.	Ventral half	WL coelom	−	
60-3	Pigeon 2 days	19 s.	Ventral half	WL coelom	−	

* Neural-crest stages based on series of normal development. For birds other than fowl unknown. For other abbreviations see Table 1.

† Dorsal half = dorsal half of neural tube, ventral half = ventral half of neural tube.

‡ MP = Mongolian Pheasant, FP = Formosa Pheasant.

without melanophores, and no donor melanophores are found in the host tissues (Table 3, cases 47-2; 47-3; 46-4; 42-8; 42-10; see Fig. 7). Ventral halves containing neural-crest cells (Fig. 9) and dorsal halves produce grafts of similar shape from which large numbers of melanophores have migrated into the peritoneum, mesenteries, skin, and feathers of the host. In some cases the graft itself was entirely free of pigment cells, all of which had apparently migrated into the host (Fig. 9).

Both the dorsal and the ventral halves of the same piece of neural tube developed in three wing-bud grafts and in one in the coelom. The dorsal produced melanophores, the ventral did not (Table 3, cases 27-6 and 7; 27-2 and 3; 42-9 and 10).

One graft of a dorsal half and one of a ventral portion were fixed in Bouin, sectioned (7 μ), and stained with haematoxylin-eosin. The dorsal half forms ganglion cells scattered through the graft, nerve cells often being arranged in short tubes and with nerve fibers and melanophores distributed peripherally (Fig. 12). The ventral part with the notochord differentiates into cartilage, nerve cells, ganglion cells, nerve fibers, but no melanophores (Fig. 13).

Ventral halves of neural tube from the region of the most posterior somites formed melanophores in four out of nine grafts. Five out of six from behind the somite region gave pigment. These results may have the following explanations. The neural tube of the more posterior levels is very thin and difficult to cut. The production of pigment by the ventral part may be due to an imperfect separation of the tissues. The following seems more probable. Since the whole neural plate has the potency to form melanophores (see p. 63), the ventral part of the neural tube must lose this potency during development. The above results may indicate that the ventral part has not yet lost this potency.

From these experiments it is concluded that pieces of the neural tube produce pigment only if they contain presumptive neural crest or recognizable crest-cells. The neural crest is the sole source of the melanophores in all the birds tested, i.e., in fowl, pheasant, robin, and pigeon.

IV. THE ORIGIN OF THE EYE PIGMENT

Kölliker in 1887 called attention to the fact that the pigment in the outer layer of the retina originates independently of branched pigment cells. This has often been used as an argument against the dominating role of the branched melanophores in the pigmentation of vertebrates. The eye of the chick contains pigment not only in the retina but also in the chorioidea and iris. The pigmentation of the retina is due to a layer of polygonal cells which produce pigment granules *in situ*, beginning during the fourth day of incubation. The pigment in the mesodermal coat of the eye vesicle (chorioidea and part of iris) is restricted to branched pigment cells, which produce pigment first at the end of the seventh day of incubation. These cells are similar to the melanophores found in the skin and mesenchyme.

The origin of the pigment in the chorioidea has been much discussed. Kölliker believed that mesenchymal cells differentiating into pigment cells invaded the chorioidea. Lewis (1903) contended that the wandering pigment cells are derived from the pigmented epithelium of the retina. Szily (1911) thought it possible that cells of the chorioidea differentiate into pigment cells. It was Bisceglie (1932) who showed that in tissue culture isolated chorioidea gives rise to mesenchyme cells and melanophores and that isolated

retina produces a layer of polygonal cells containing melanin granules but never branched pigment cells.

Further evidence for the difference between the retinal pigment cells and the melanophores is provided by the character of the eye pigmentation in the White Leghorn. In this breed Dorris (1936, 1938) found that explants from embryos containing neural crest formed melanophores in tissue culture. In a special study of the normal White Leghorn embryo I have found a number of melanophores also in the skin and beneath the coelomic epithelium after the seventh day of incubation. At the time of hatching, the skin melanophores accumulate at the base of the down feathers, leaving the epidermis almost colorless as in most darkly pigmented breeds. The White Leghorn melanophores contain small dark granules, but only few of these cells are filled with them. Colorless granules are more abundant. All these melanophores migrate into the feather germs.

TABLE 4*

THE DIFFERENCE IN PLACE OF ORIGIN OF RETINAL AND CHORIOID PIGMENT

Case No.	Donor	Implant	Neural Crest† Stages	Host	Retinal Pigment	Melanophore Production
48-2	BR 6 s.	Left optic vesicle	Crest mid-brain level	WL coelom	+	−
48-4	BR 6 s.	Left optic vesicle plus midbrain	Migrating cells in midbrain	WL coelom	+	+
48-7	BR 8 s.	Distal part of eye vesicle	Migrating cells in midbrain	WL coelom	+	−
48-12	BR 11 s.	Right forebrain with optic vesicle	Crest in fore-brain level	WL coelom	+	+
48-9	BR 12 s.	Distal part of eye vesicle	Beginning cell migration at forebrain level	WL coelom	+	−
48-10	BR 12 s.	Distal part of eye vesicle	Beginning cell migration at forebrain level	WL coelom	+	−

* For abbreviations see previous tables. † Neural-crest stages based on normal development.

The fact that the number containing black granules is small is probably one of the chief factors causing the "whiteness" of White Leghorn feathers. In other words, a small quantity of pigment is distributed over a wide area.

The retinal pigment in the White Leghorn embryos is as dark and abundant as in a dark breed. But the chorioidea contains a small number of branched pigment cells with as few black granules as in those found in the skin. This striking difference in the pigment formation is further evidence for the independent origin of retinal and chorioid pigment.

To determine whether retinal cells may give rise to branched pigment cells, pieces of the eye cup of a 72-hour White Leghorn embryo were grafted into the wing bud of a 70-hour White Leghorn host. At hatching the graft had formed a small rounded clump of retinal epithelium at the base of the host wing. No dark cells had migrated out into

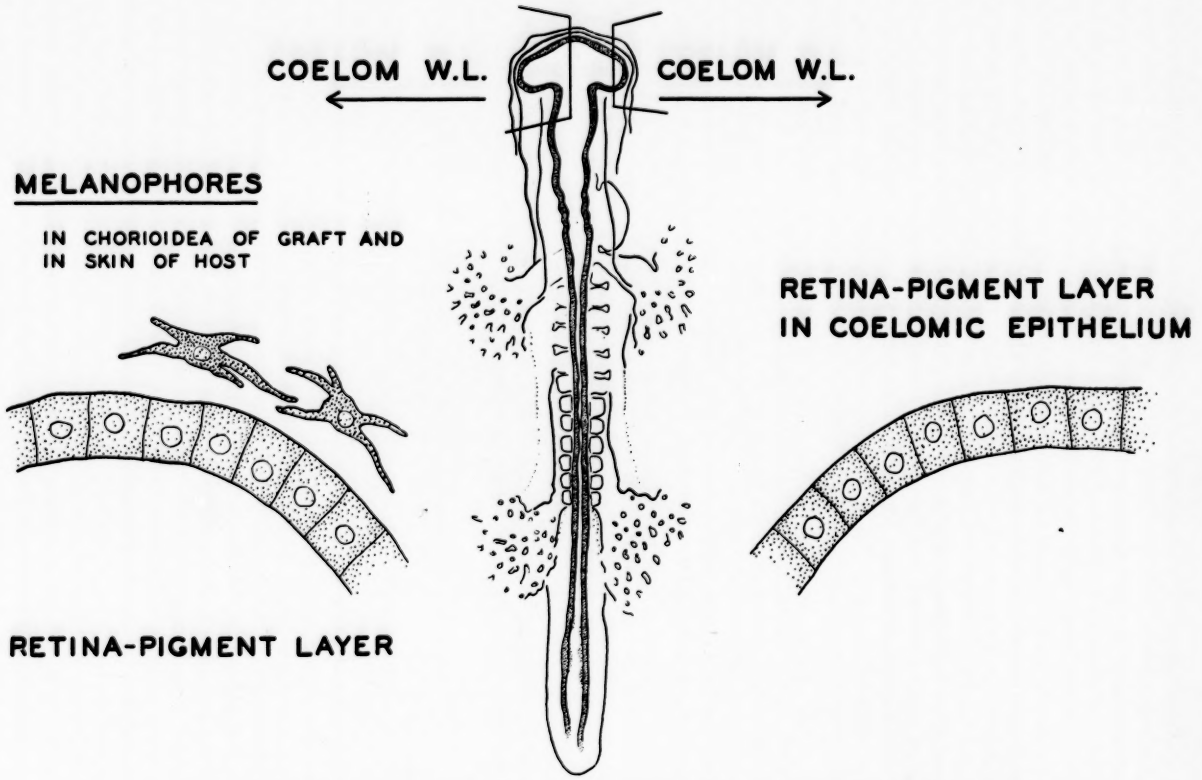

FIG. 17.—Diagram showing relationship of retinal and chorioid pigment to the neural crest (see text and Table 4)

the host. This indicates that retinal epithelium is not capable of producing melanophores. This is confirmed by the result of the next experiment.

Another problem is that of the origin of the two types of pigment cells. The similarity of the chorioid melanophores to those of the skin suggests their origin from the neural crest. What then is the relation of the retinal pigmentation to the neural crest? To answer these questions two types of experiments were carried out (Fig. 17): (1) Eye vesicles of Barred Rock embryos from 6-12 somites were isolated before the neural-crest cells could have migrated in and transplanted into the coelom of 60–70-hour White Leghorn embryos. (2) Eye vesicle with forebrain wall of similar embryos, containing neural crest, were transplanted in the same manner.

The results are given in Table 4. When the optic vesicle does not contain neural-crest cells, it forms a clump of tissue with folded retinal pigment epithelium. No melanophores, however, are found (Table 4, cases 48-2; 48-7; 48-9; 48-10). On the other hand, if the graft contains neural-crest cells, a great number of melanophores is observed in the chorioidea of the grafted eye which developed in the coelom, as well as in the peritoneum, skin, and feathers of the host (see cases 48-4; 48-12; and Fig. 10).

The retinal and chorioid pigments in the chick are therefore shown to be independent in origin as demonstrated already in tissue culture by Bisceglie and in our coelomic grafts. One of them may be suppressed while the other one is normally produced as we find it in the White Leghorn. The chorioid pigment cells, characteristic melanophores, are derivatives of the neural crest. The retinal-pigment epithelium, on the other hand, arises in absence of crest cells.

DISCUSSION

I. ORIGIN OF MELANOPHORES

Until recently all theories dealing with the origin of melanophores have been based merely on histological pictures or on the Dopa reaction. It is clear that these methods can reveal only the fate of the pigment cells after the formation of the pigment or the presence of oxidizing enzymes. Any migration of cells before they can be distinguished by these methods was thus left out of consideration. Only the experimental method of isolation and transplantation can test inherent capacities for autonomous pigment formation in the embryonic tissues. This method led to the discovery of the origin of the melanophores from the neural crest in amphibia by Du Shane and in birds by Dorris and by Eastlick and is basic to this work.

Dorris (1936 and 1938) gave evidence from *in vitro* experiments that the neural crest is the source of melanophores in birds. In 1939 she grafted pieces of "neural crest" of black or white breeds into the leg bud of 72-hour embryos. Although the results of these experiments gave strong evidence for the neural-crest origin of the melanophores, sufficient proof was not given. Indeed, neither the development of the neural crest nor its relationship to the production of melanophores in the grafts was discussed. Controls showed only that the pigment was not produced in epidermal or mesenchymal cells. Furthermore, the possibility was left open that other parts of the neural tube might give origin to melanophores. It is impossible to separate the irregular cell strands of the neural crest from the neural tube by the methods used. The grafted pieces, therefore, were not pure neural crest. Consequently, their ability to form pigment is not proof for the derivation of the melanophores from the neural crest alone. Lastly, the method of

grafting in the leg bud might be objected to, since a negative case may be due only to a lack of growth of the transplant.

To give sufficient proof that only the neural crest gives rise to melanophores their appearance or absence in transplants would have to be correlated with the morphological development of the neural crest at different ages and at different anterior-posterior levels. It had to be demonstrated that only tissues containing presumptive neural crest, histologically recognizable neural crest, or cells migrating out of the neural crest can produce melanophores. Tissues grafted into the coelom develop into bodies which can be analyzed histologically. Therefore, it was certain in every case whether or not the graft had developed and produced pigment. In the foregoing pages these conditions have been met, and it was shown that the lateral migration of the melanophore-producing potency coincides with the development of the neural crest and that only tissues containing neural crest can produce pigment.

Next the question of the origin of the buff and red-brown pigments arises. These granules as well as the black ones are produced in branched cells, one cell forming only granules of a specific shape and color (Danforth, 1937; Krätzig, 1937). Grafts from Brown Leghorn, pheasant, and robin embryos containing no neural-crest cells give white feathers, whereas grafts containing neural crest always give melanophores characteristic of the donor. These data show that the melanophores producing buff and red-brown granules are derived also from the neural crest. Whether in species having more than one type of melanophores they are different cells, early differentiated, or whether the surrounding tissues, skin, and feather germs or hormones (Krätzig, 1937) influence the kind of pigment they produce, is not yet known (see summary in Fig. 18).

II. MIGRATION OF NEURAL-CREST CELLS AND MELANOPHORES

It has been known for some time that neural-crest cells have the capacity to migrate throughout the body of the embryo, giving origin to such structures as spinal nerves, sheath cells, etc. That the melanophores also have the ability to wander through tissues is made probable by their amoeboid movement which has been observed directly in tissue culture (Dorris, 1936, 1938) and indirectly by the spreading of donor pigment cells in wing-bud grafts (Willier, Rawles, and Hadorn, 1937; Willier and Rawles, 1938, 1940; Rawles, 1939). This migration takes place chiefly in loose tissue, which may explain the great number of melanophores in the mesenchyme. Furthermore, they appear to follow along the surface of dense structures. Neural-crest cells can be seen on the dorsal surface of the dermatome and along the lateral walls of the neural tube, and the fact that melanophores are distributed abundantly along blood vessels has been pointed out throughout the literature. They are often found gathered around them, particularly along the capillaries. This can be seen nicely beneath the coelomic epithelium of a Barred Rock at hatching. But nerves also are followed. This tendency is strikingly shown in those cases where grafts from pigmented breeds produced melanophores in the coelom of White Leghorn chicks. The melanophores, having left the graft, migrate into the peritoneum, accumulate around blood vessels, nerves, and urinary and sex ducts and follow these structures a long way in the host.

In the chick of a black breed at hatching we find a distinct line in the umbilicus where the melanophores seem to accumulate but never pass. A section of this part shows a sudden change in the arrangement of the mesenchyme cells. In the body wall and mesenteries these cells are nearly parallel to one another. At the border to the umbilicus

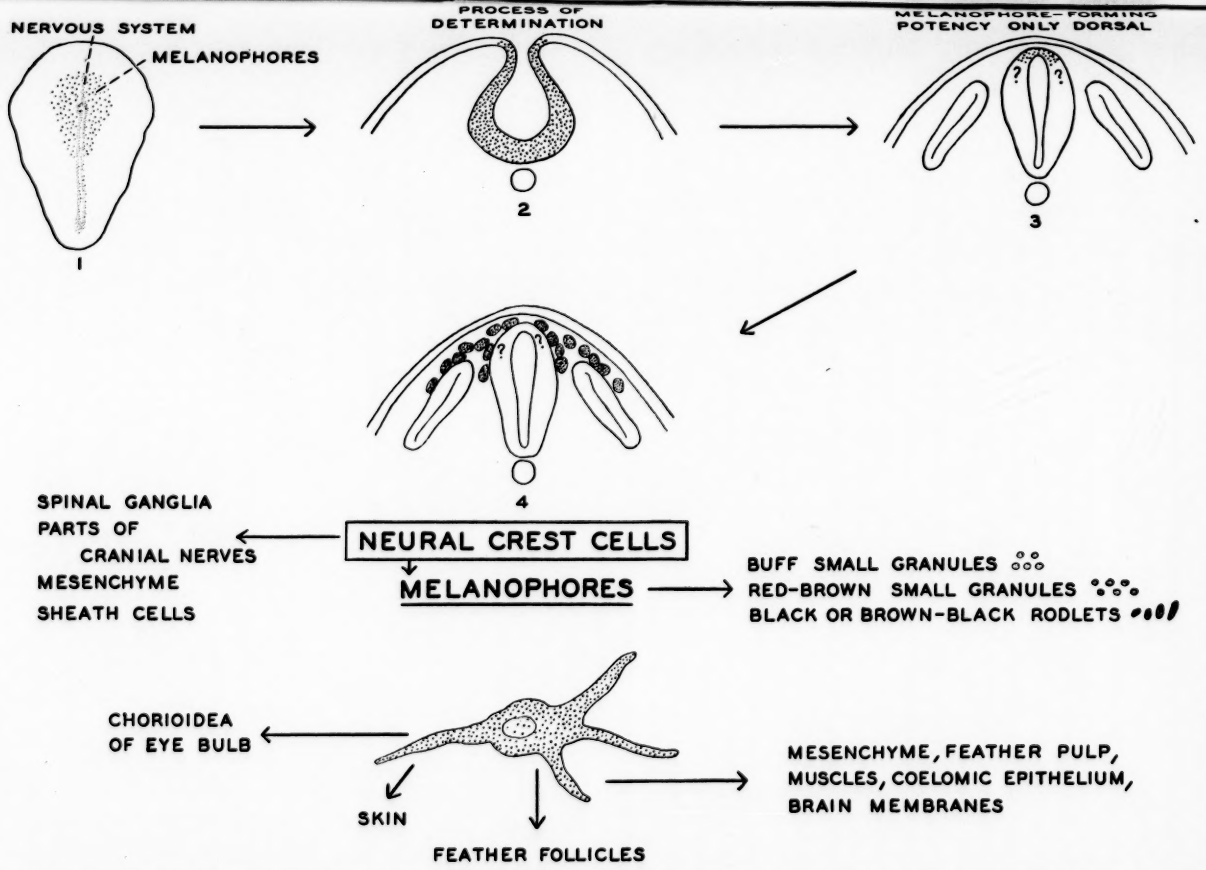

FIG. 18.—Diagram showing the gradual restriction of the melanophore-producing potency. 1. Head-process stage. The melanophore-producing potency coincides with area forming nervous system. 2. During closure the whole neural tube has melanophore-producing potency. 3. The potency to produce melanophores is restricted to the roof. 4. The melanophore-producing potency is localized in the migrating neural-crest cells.

they become very irregular, crossing in all directions and apparently forming a barrier to the migration of the melanophores. In explaining unpigmented regions, we must therefore consider the possibility of mechanical hindrances to the migration of pigment cells.

Eastlick (1938 and 1939*b*) found that grafts in a pigmented embryo were free of host melanophores if attached to the mesentery, from which he concluded that the melanophores did not cross this structure. Similar experiments and an investigation of the pigmentation of chicks at hatching throw more light on this problem and lead to the following results: (1) The mesenteries in Brown Leghorn, Barred Plymouth Rock, and F_1 hybrid in a hatching chick do not contain melanophores. Therefore, an unpigmented graft attached only to the mesentery of the pigmented host remains free of melanophores. (2) Melanophores are able to migrate into the mesentery, if pigmented grafts are attached to them (Fig. 9). Skin and coelomic epithelium, however, remain free of donor pigment in these cases. (3) Donor melanophores migrate into the peritoneum and skin only if a melanophore-producing graft is attached to the body wall. They appear in the mesenteries only if the graft is attached to them. Often a graft is attached to both, leading to a donor pigmentation of skin, peritoneum, and mesentery. From these observations the conclusion is reached that something must inhibit the migration of melanophores into the mesentery during normal development. The same factors hinder the melanophores from leaving the mesentery when provided with a pigmented graft. Comparing this with the situation in the umbilicus suggests a mechanical barrier, i.e., the arrangement of cells prevents the migration of melanophores.

Another problem to consider is the direction of migration of the neural-crest cells. Do they migrate only laterally or also in a longitudinal direction? In Barred Plymouth Rock embryos (50 hours) the neural tube was cauterized over a length of several somites. Three chicks so treated hatched and showed a dorsal opening of about 10 mm., where the spinal cord was destroyed. Yet the skin and mesenchyme all around contain melanophores, demonstrating that the neural-crest cells migrated also in a longitudinal direction. This possibility has to be considered in those experiments where extirpations of the neural crest over a certain length are made to prove the crest origin of any cell type.

It has been suggested that the spread of pigment in embryonic grafts from pigmented to unpigmented breeds and the gradual lateral expansion of the pigment-producing agent in the developing embryo might be due to a diffusion of some substance necessary for pigment production. No evidence supporting this hypothesis has been found. To the contrary, the following facts show that the spreading is due to a migration of pigment-producing cells: (1) The fact that grafts develop on the chorio-allantois or in the coelom of pigmented breeds without producing pigment excludes the presence of such a substance in the blood stream. (2) Except in the retina all melanin is produced in a specific type of branched cell having the ability to migrate through the tissues. (3) These cells are shown to be, in all probability, derivatives of neural-crest cells. The lateral spread of the melanophore-producing agent corresponds to the outgrowth of cells from the neural crest. (4) In grafts from one breed of chick to another and from pheasant, pigeon, and robin to chicks pigment granules peculiar to the donor are formed in melanophores. It is very improbable that a "diffusing substance" could produce donor-specific granules in host cells. (5) An irregular arrangement of mesenchyme cells apparently forms a mechanical barrier for the spread of pigmentation. It is hardly possible that this could be a hindrance for a diffusible substance.

III. PIGMENT FORMATION AND NERVOUS SYSTEM

Rawles and Willier (1939) and, independently, Eastlick (1939*a*) demonstrated that in primitive-streak and head-process stages the area producing nervous system in chorio-allantoic grafts coincides with that giving rise to melanophores. In later development Hensen's node always gives pigment in grafts (Watterson, 1938), as also do portions of the most posterior neural tube, as shown in the foregoing pages. As development proceeds, the ventral part of the neural tube loses the melanophore-producing potency. Whether this determination is due to a separation and migration of cells or to an actual change in the cells is difficult to decide.

In all these experiments with different birds and with all types of melanin (buff, red-brown, and black)—contrary to the statements of Klee (1889), Lloyd-Jones (1915), and Greite (1934)—no evidence was found for the production of melanophores in epidermal or mesenchymal cells. Only derivatives of the nervous system can give origin to melanin, viz., the retinal-pigment layer and the melanophores derived from the neural crest.

In mammals the origin of the melanophores (melanoblasts of Bloch) from the neural crest is very probable but has not yet been demonstrated. The more recent investigators assume that epidermal cells form melanin independently of the branched pigment cells. But all their evidence is based on purely histological studies and the Dopa reaction. Whether the epidermal cells of mammals are really able to produce melanin pigment independent of melanophores can be established only by early isolation and transplantation of mammal skin and mesenchyme.

IV. THE HOMOLOGY OF VERTEBRATE MELANOPHORES

Branched melanin-producing cells have been observed in the epidermis and in many mesodermal structures of all the vertebrate groups. Their morphological similarity is striking. They all possess the capacity to migrate, and their invasion into the epidermis from the mesoderm has been described for all classes. In cold- and warm-blooded groups—viz., amphibians and birds—their origin from the neural crest is now demonstrated. Yet one great difference exists between the lower and the higher vertebrates. Fishes, amphibians, and reptiles are able to change their color owing to the movement of the chromatophores and of the pigment granules in the cells themselves. The pigment is carried by living cells which are influenced by nervous and hormonal stimulations, by temperature and by light. The skin of birds and mammals, however, is covered by dead epidermal structures—feathers and hair which serve as temperature insulators. Movements of melanophores in the skin would give no effect on the appearance of the coats of warm-blooded vertebrates. Yet in mammals the melanophores are still approached by nerve fibers as Sasybin (1933) has shown. Whether this holds true for birds has yet to be demonstrated. The difference between the melanophores in lower and in higher vertebrates seems to be due, therefore, largely to the fact that dead cells of hairs and feathers affect the coloration in birds and mammals. Their agreement in form, in melanin-production relationship to the nervous system and hormones, in migration, and, above all, their origin from the neural crest (amphibians and birds) leads to the conclusion that they are homologous cells. In view of this homology the same name should be applied to these cells in all classes. In lower vertebrates they are usually called melanophores—the melanin-producing type of chromatophore. Bloch (1930) called them melanoblasts in mammals to distinguish them from another type of cell—the

melanophores which he thought unable to produce melanin but capable of carrying it over from melanoblasts to epidermal cells. Since the existence of such cells is still hypothetical, the older name "melanophore" has been given preference in this work.

If we are allowed to generalize from the conditions obtaining in amphibians and birds we reach the conclusion that in all groups of vertebrates the melanin pigmentation in its great variety is produced by one type of cell—the melanophores. These are derived from the neural crest and migrate into the various pigmented parts of the animal, particularly the skin and its structures.

SUMMARY

1. By isolating and transplanting portions of various pigmented fowl and other bird embryos from different levels along the embryonic axis to the wing bud, coelom, and chorio-allantoic membrane of host chick embryos it is demonstrated that (1) the melanophores originate outside of the epidermis or mesenchyme, migrating into them at a certain stage of development, and (2) only tissues containing presumptive neural crest or neural-crest cells which have migrated into them (as revealed by histological study) give rise to melanophores when transplanted to White Leghorn embryos. Thus, if isolated early enough, i.e., before the neural-crest cells migrate in, grafts from pigmented breeds develop skin and feathers without pigment.

2. By a histological study of the control side of the donor embryo it has been possible to correlate the appearance of melanophores in the graft with the morphological development of the neural crest at the time the isolation was made.

3. By dividing segments of the neural tube into dorsal and ventral halves and transplanting each separately into the wing bud or coelom of White Leghorn host embryos, it is shown that (1) the dorsal halves containing either presumptive or morphologically observable neural crest give rise to melanophores and (2) the corresponding ventral halves do not produce melanophores.

4. Experiments were made to determine the origin of the retinal and chorioid pigments of the eye and their relationship to the neural crest. The pigment of the retina is produced in every cell of the pigmented layer and is not derived from the neural crest. The chorioidea contains pigment in characteristically branched cells, the melanophores, which are identical with those in the skin and mesoderm and, like them, derived from the neural crest.

5. From the experimental evidence now accumulated for various pigmented fowl—pheasant, Black Fantail Pigeon, and robin—it is highly probable that all melanin-producing cells are derived from neural-plate material—the retinal pigment from the forebrain, the melanophores from migrating neural-crest cells.

6. The possible homology of the melanophores in all vertebrate groups, indicated by their agreement in development and by their morphological and physiological characters, is discussed.

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PLATE I

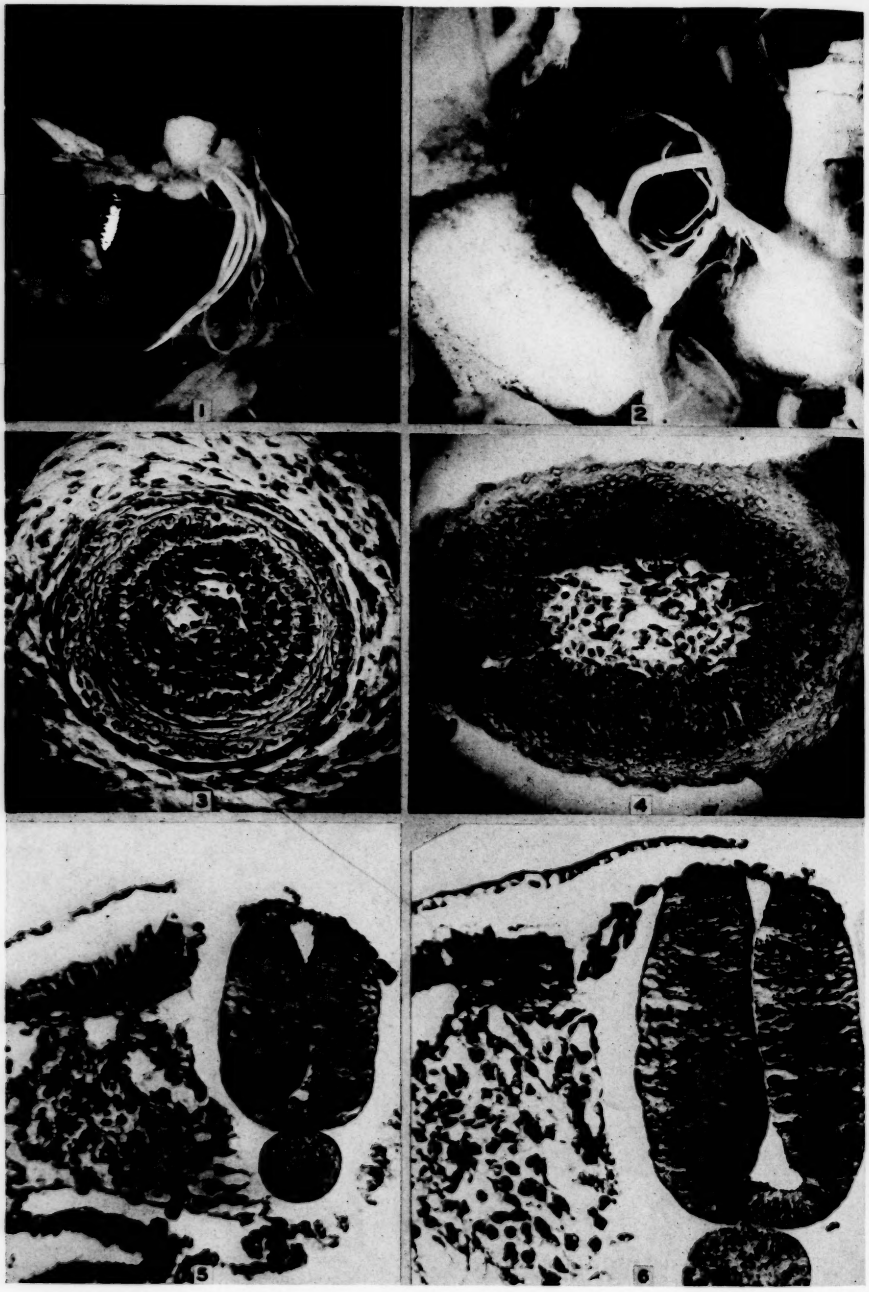

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PLATE I

FIG. 1.—Coelomic graft with white down feathers, obtained from a piece of lateral plate of a 16-somite Ringnecked Pheasant, cut between neural tube and somites and implanted into the coelom of a White Leghorn. No melanophores developed (case 55-6).

FIG. 2.—Vesicle of skin containing black and white down, grown in the coelom of a White Leghorn host, derived from head skin of a Barred Plymouth Rock embryo of 37 somites. Note on the left side the donor melanophores in the body wall of the White Leghorn host (case 7-2).

FIG. 3.—Cross-section through a feather germ of the transplant shown in Fig. 1. No melanophores present. Bouin, haematoxylin-eosin.

FIG. 4.—Cross-section through a feather germ developed from a piece of lateral plate at the level of the twenty-fifth somite of a Barred Rock embryo of 31 somites, grafted to the chorio-allantois of a Barred Rock embryo. Note the presence of melanophores (case 34-8).

FIG. 5.—Cross-section of left side of donor Barred Plymouth Rock embryo of 23 somites at the level of the tenth somite. The corresponding right side was cut at the edge of the neural tube, thus excluding neural-crest cells, and grafted to the chorio-allantois of a White Leghorn. No melanophores developed in the graft (case 39-2.) The cutting caused the slight distortion seen on the right side of the tube.

FIG. 6.—Cross-section of left side of donor Barred Plymouth Rock embryo of 31 somites at the level of the twenty-fifth somite. The corresponding right side was cut between the neural tube and the somites, thus including migrating neural-crest cells, and grafted to the chorio-allantois of a Barred Plymouth Rock. The graft developed melanophores (case 34-8).

PLATE II

FIG. 7.—Intracoelomic graft (*G*) developed from the ventral half of the neural tube of a Barred Rock of 18 somites at the level of the fourteenth somite. Note the absence of melanophores (case 47-3).

FIG. 8.—A piece of neural tube from a Barred Rock of 13 somites developed in the coelom of a White Leghorn host. The implant was cut at the level of the sixth somite. Note the melanophores in the graft (*G*) and the body wall of the host (case 20-5).

FIG. 9.—Intracoelomic graft (*G*) from ventral half of the neural tube of an F_1 hybrid of 30 somites isolated at the eleventh somite level. The ventral half at this level contained neural-crest cells. Note the melanophores which migrated into the mesentery. No melanophores were found in the body wall or skin of the White Leghorn host (case 40-8).

PLATE II

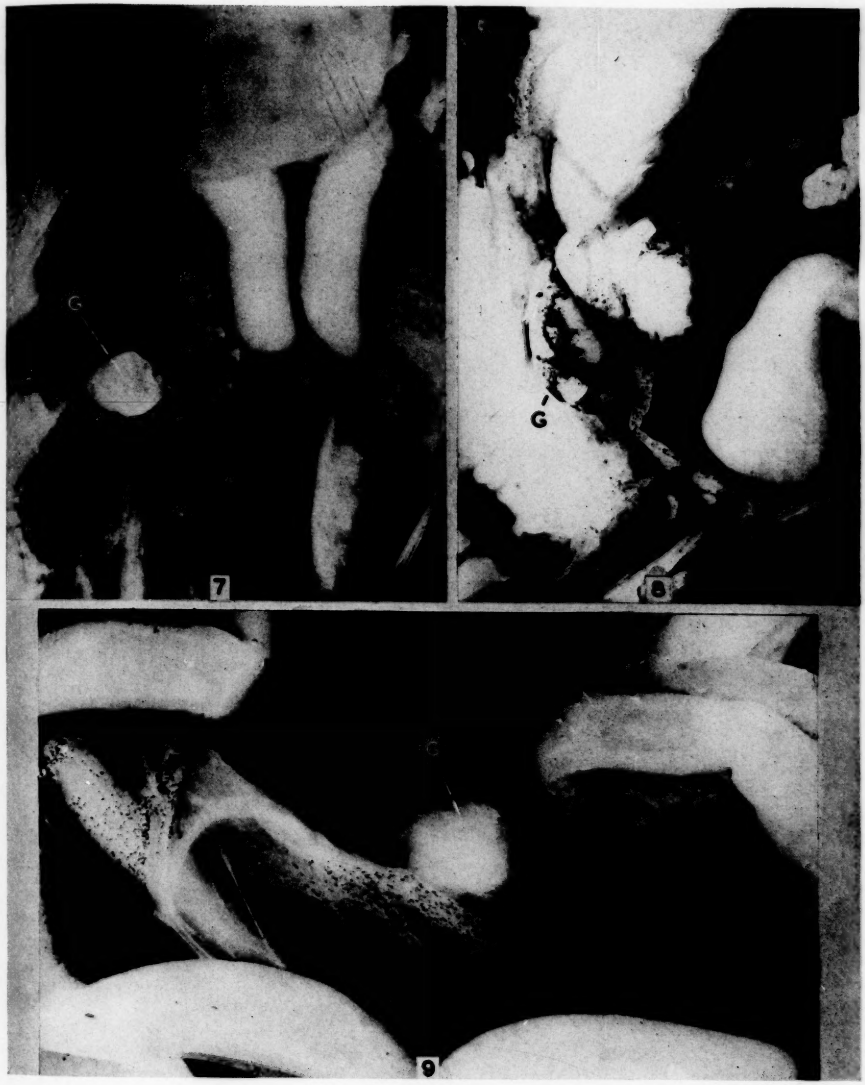
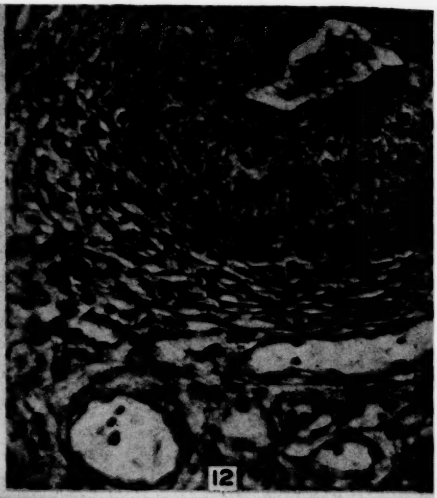


PLATE III



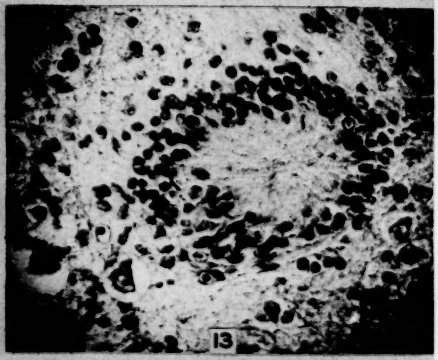


PLATE III

FIG. 10.—Abnormal eye vesicle (*E*) with pigmented layer of retina and pigmented chorioid coat grown in the coelom of a White Leghorn. The implant contained one side of the telencephalon plus the corresponding optic vesicle of a Barred Rock of 11 somites. The chorioidea contains melanophores, some of which have also migrated into the body wall of the host (case 48-12).

FIG. 11.—Epithelium of retina with black pigment developed from a piece of an eye bulb of a Barred Rock of 8 somites in the coelom of a White Leghorn embryo. No melanophores present (case 48-7).

FIG. 12.—Section of a graft from the dorsal half of the neural tube, cut at the level of the eighth somite of a Barred Plymouth Rock of 35 somites and implanted into the coelom of a White Leghorn. Note neural tube (?) and melanophores in mesenchyme below (case 42-2).

FIG. 13.—Section of a coelomic implant developed from the ventral half of the neural tube of a Mongolian Pheasant of 26 somites cut at the level of the twenty-fourth somite. Note mass of ganglion cells, nerve fibers, and the absence of melanophores (case 56-5).

FIG. 14.—Section of a coelomic graft obtained from a piece of an eye vesicle of a 6-somite Barred Rock. Note retinal pigment and absence of melanophores (case 48-2).

THE KARYOPLASMIC RATIO IN THE EMBRYOS OF MONOVOLTINE AND BIVOLTINE RACES OF THE SILKWORM BOMBYX MORI L.

(Four figures)

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RACES of *Bombyx mori* L. are known which have one generation a year (monovoltine), two (bivoltine), or several *generations* (polyvoltine). The monovoltine moths lay eggs in summer that first show a period of embryonic development of 2–3 days prior to the formation of the embryonal stripe, the serosa, and the amnion. The monovoltine eggs remain in this stage during late summer, autumn, and winter; the following spring they resume their development and hatch. Under normal conditions the period of morphological quiescence continues 9 months or more. While experimentally this may be shortened or prolonged, it is always necessary for the eggs to pass through a period of low temperature—but not longer than 2 or 3 months—at 6°–7° C.

The development of polyvoltine eggs is continuous; they hatch within 10–20 days after oviposition, according to the temperature. The polyvoltine races are present in tropical countries.

The cells of the serosa of the monovoltine eggs are charged with pigment, while those of the polyvoltine are not pigmented. The bivoltine races have the first generation in the spring, laying eggs that develop immediately. The serosa of these is without pigment, as in the case of polyvoltine eggs. The moths produced from such eggs lay eggs that develop as do those of the monovoltine races, having pigmented serosa, a period of morphological quiescence during autumn and winter, and requiring a period of low temperature. All monovoltine races are more or less variable; some eggs develop as do bivoltine races, while the reverse may be observed in the bivoltine laying.

The phenomenon of monovoltinism and bivoltinism has been the subject of extensive investigations. Some authors believed that the phenomenon was strictly due to the climate, so that the monovoltine races transferred from temperate to tropical climates became bivoltine, and vice versa; but monovoltinism, bivoltinism, and polyvoltinism do not now appear so easy to explain, and workers are not yet in agreement regarding the meaning of all the observed phenomena. It is known with certainty only that some environmental conditions do facilitate bivoltinism and polyvoltinism and some do not; that each race may shown an internal tendency to various forms of voltinism;[1] and that the environmental conditions may produce different effects according to the vital stage of the insect, i.e., high temperatures during incubation are probably opposed to bivoltinism and favorable to monovoltinism (Mollo, 1921, mentioned by Foa, 1927). Actually, we may say that the tendency to bivoltinism or monovoltinism is also dependent upon internal conditions that act in harmony with the environmental conditions determining the degree of voltinism.

[1] From a bivoltine race Foa (1932) was able to select a monovoltine race.

In this work a continuance of the author's investigations on the physiology of *B. mori* L., a fact that may be connected with the biological mechanism of voltinism, is made known.

Foa (1927), studying the results of Kiyoshi Katsuki (1918), observed that this author found the nuclei of the germinal cells in a bivoltine race of greater volume than those of a monovoltine race; Foa thought in consequence that the karyoplasmic ratio must be different according to the voltinism. She examined the serosa of various races but because of technical difficulties was unable to observe differences in the volume of the nuclei. Scatizzi (1936) confirmed the difference in volume between the monovoltine and the bivoltine germinal cells.

In "Chinese white," "Italian yellow" (monovoltine), and "Awoiko" (bivoltine) Foa (1927) observed that the embryonal stripe (germ band) of bivoltine eggs has larger nuclei (in a simple microscopical observation), the nuclei of the monovoltine Chinese white being smaller and those of the Italian yellow the smallest, though she did not measure the exact size of the cells and of the nuclei. The present author wishes to explain more fully the problem of measuring the nucleoplasmatic quotient of the embryonal stripe of monovoltine and bivoltine races.

MATERIAL AND TECHNIQUE

The author employed monovoltine eggs of Italian yellow race from Padua and bivoltine eggs of Awoiko race from moths bred in Portici (Naples). The eggs were fixed with Carnoy, the Carnoy and the chromoacetic mixture being probably the best fixing liquid for the eggs with the chorion.

The shells were removed from the fixed eggs with Rusma (Niceta, 1929). They were then imbedded in paraffin, cut, and the sections (5 μ) stained with haematoxylin Carazzi and eosin. For measurement the author selected the best sections. The eggs examined were in diapause, before wintering.

Landi (1934) measured the karyoplasmic ratio of silkworm eggs during incubation and the total nitrogen; the karyoplasmic ratio has been expressed by Landi by the formula $NP \times 100/NT - NP$, in which NP is the nitrogen of the aminopurinic bases and NT the total nitrogen. This quotient increases during incubation from 2.30 to 2.96–3.06 at the close of the incubation period.

The karyoplasmic ratio measured by Landi is not comparable to that obtained by measuring the nuclear and cellular volumes, because Landi analyzed the entire contents of the eggs, including the yolk.

RESULTS[2]

The cells of embryonal stripes (germ bands) have nuclei with a visible membrane, chromatin divided in granulation, and cytoplasm lightly granular.

The measurements were made either directly with a micrometric ocular or indirectly from photomicrographs of some good sections at the same magnification.

The volume of the nuclei has been calculated as follows. The ectodermal and mesodermal nuclei are very clearly delimited and appear as elipsoids; the two diameters of these were measured, and the nuclear volume was then calculated.

[2] The earlier results of my investigations on the karyoplasmic ratio were privately announced to Dr. Foa, who kindly published my letter in her work of 1937.

In practice the author calculated the volume of an elipsoid for each stripe, the diameters of which were the average of the diameters of all the nuclei observed (i.e., the greater diameter was the average of the greater diameters of all the nuclei; the smaller diameter was the average of the smaller diameters). The volume of the ectodermal and mersodermal nuclei was calculated separately. Finally, the author obtained the average of the average nuclear volumes of each stripe.

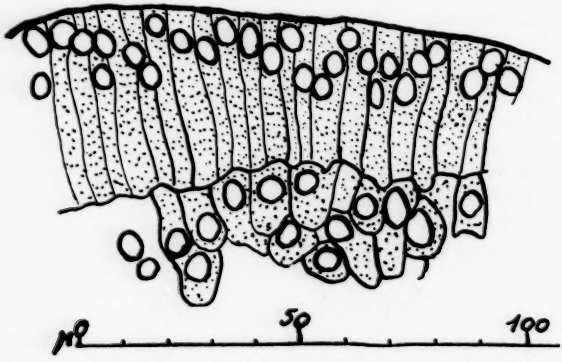

FIG. 1.—Semischematic drawing of a region of the embryonal stripe of an Italian yellow egg in diapause. From a photomicrograph.

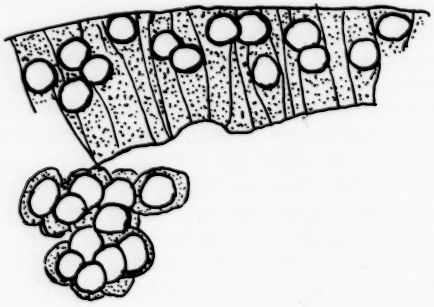

FIG. 2.—Semischematic drawing of a region of the embryonal stripe of an "Awoiko" egg (bivoltine) in diapause. From a photomicrograph. (The drawing and photomicrographs are all made with the same magnification. Cf. the scale of Fig. 1.)

The volume of the ectodermal cells was indirectly calculated as if cylindrical, taking as their diameters the smaller diameter of the ectodermal nuclei and as their length the height of the stripe. It may be clearly observed in Figures 1–4 that the cellular boundaries are often tangent to the nuclear boundaries. The cells are really not regular cylinders, but in the eggs observed the ectoderm is formed by a monostratified epithelium. The length of the cells occupies the entire height or thickness of the stripe, and the nuclei generally appear in a line toward the amniotic cavity. Some ectodermal nuclei appear

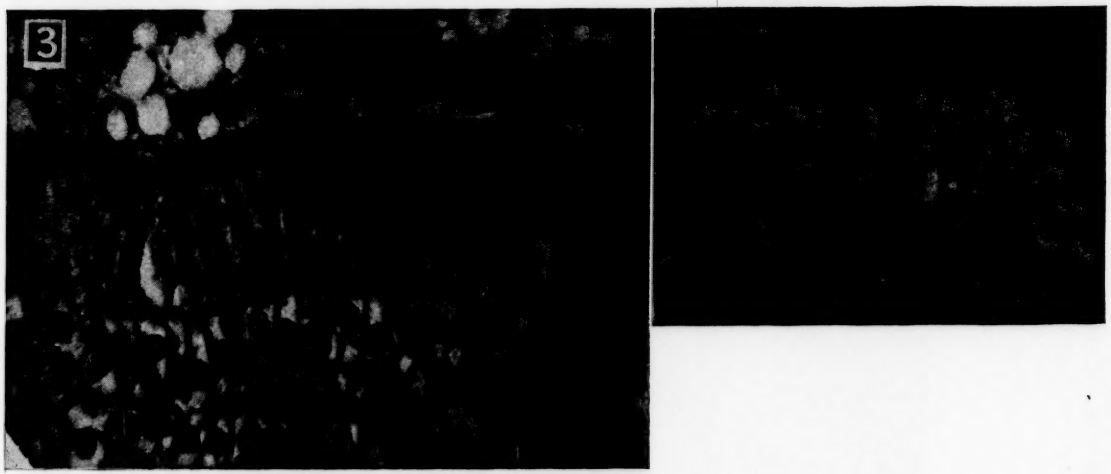

FIG. 3.—Photomicrograph of cells of the embryonal stripe of a monovoltine race (Italian yellow)

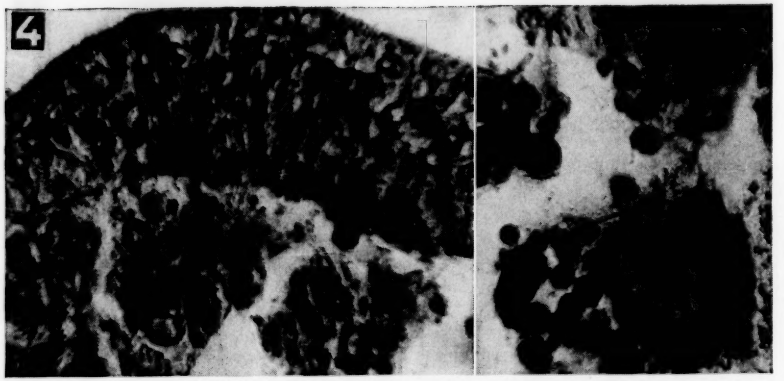

FIG. 4.—Photomicrograph of cells of the embryonal stripe of a bivoltine race (Awoiko). Observe the smaller volume of the nuclei compared with those of Figure 3.

toward the mesoderm; but it may also be observed that the corresponding cells extend for the total height of the stripe, from the mesoderm to the amniotic boundary.

The nuclear volume has been compared to the volume of the entire cell or to the volume of the cell diminished by the nuclear volume. The mesodermal cells are some-

TABLE 1

THE NUCLEOPLASMATIC RELATIONSHIP

A. SIZE AND VOLUME OF THE NUCLEI

Nuclei	Smaller Diameter	Greater Diameter	Volume
Monovoltine:			
Ectodermal	4.78 μ	5.96 μ	71.24 μ^3
Mesodermal	4.83	5.62	68.74
Bivoltine:			
Ectodermal	6.79	8.42	205.50
Mesodermal	6.80	8.79	212.75

B. SIZE AND VOLUME OF THE CELLS

Cells	Average Height	Average Width	Volume of Total Cell	Volume of Cytoplasm
Ectodermal:				
Monovoltine	36.89 μ	4.78 μ	1029.1 μ^3	958.0 μ^3
Bivoltine	35.8	6.79	1298.49	1093.0
Mesodermal:				
Monovoltine	13.07	13.07	1168.61	1099.87
Bivoltine	12.00	12.00	904.61	691.85

C. RELATIVE VOLUME OF THE NUCLEI
(Karyoplasmic Ratio)

	Cell Volume	Nuclear Volume	Volume of Cytoplasm	Nuclear Volume
Monovoltine:				
Ectoderm	100	6.92	100	7.43
Mesoderm	100	5.88	100	6.24
Bivoltine:				
Ectoderm	100	15.82	100	18.8
Mesoderm	100	23.51	100	30.75

what irregular in form although roughly spherical, and their volume was calculated by considering the cells as spheres. Two or more diameters of each cell were measured, and the volume was calculated with the average of the various diameters.

The diameter of the mesodermal cells is about 2.5 times greater than the average diameter of the mesodermal nuclei in the Italian yellow; in the bivoltine eggs the

diameter of the mesodermal cells is about 1.65 times that of the average diameter of the mesodermal nuclei. The general results are summarized in Tables 1 and 2.

The mesodermal bivoltine cells have very little cytoplasm. In some stripes the nuclei of these cells appear as if surrounded by only a fine stratum of cytoplasm. Further

TABLE 2

CELL MEASUREMENTS

Number	Nuclei (Average Diameter)		Embryonal Stripe
	Ectoderm	Mesoderm	Average Height of Cells
	Embryonal Stripe, Monovoltine (Italian Yellow "Ascoli")		
1	5.58	5.35	39.62
2	5.20	4.51	38.55
3	5.96	5.58	42.53
4	5.27	4.36	30.90
5	5.27	4.89	33.35
6	4.74	.....	41.31
7	5.50	5.04	37.79
8	5.04	.....	40.69
9	5.27	.....	.....
10	5.27	5.81	35.03
11	5.35	5.20	37.63
12	5.04	.....	32.13
13	5.73	5.20	35.34
14	5.81	4.89	31.05
15	5.27	5.43	30.75
16	5.35	5.66	33.50
17	4.81	4.05	40.23
18	5.66	6.04	40.69
19	6.04	5.96	38.25
20	5.12	5.73	38.55
21	5.35	5.12	39.93
	Embryonal Stripe, Bivoltine (Awoiko)		
11	8.01	8.38	.....
12	7.19	7.43	31.93
23	8.12	7.68	.....
24	6.42	7.12	38.43
26	7.75	8.39	37.22

measurements with a more exact method, such as comparing with a planimeter the cellular and nuclear surfaces, should give absolute results slightly different from those indicated; but the relative results will not be changed materially.

Table 2 also indicates that the form of the nuclei is variable; the mesodermal nuclei are more rounded than the ectodermal in monovoltine eggs. The ectodermal nuclei,

moreover, may be more or less elongated according to the eggs. In the bivoltine eggs examined the ectodermal and mesodermal nuclei appear more rounded than the corresponding nuclei of the monovoltine eggs; in bivoltine eggs the author has not observed such a considerable difference in form between the mesodermal and the ectodermal nuclei of the monovoltine stripe.

In the bivoltine eggs we have also observed one or two very large nucleoli; in monovoltine eggs the nucleoli are smaller or absent. A nuclear and cellular variableness may also occur in the same stripe. Not only is the karyoplasmic ratio greater in the bivoltine races, but so is the absolute volume of the ectodermal and mesodermal cells, in comparison with the monovoltine races. We may suppose that the higher metabolism of the bivoltine races is correlated with the relatively greater volume of the nuclei.

SUMMARY

In the embryonal stripe (germ band) of a monovoltine race of *B. mori* L. (Italian yellow Ascoli) the karyoplasmic ratio is greater during the diapause than in a bivoltine race (Awoiko) in both the mesodermal and the ectodermal cells. There is also variability in form and volume of the mesodermal and ectodermal nuclei in the eggs of each race considered.

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THE ROLE OF THE INJURED SURFACE IN THE PRODUCTION OF THE INJURY POTENTIAL OF FROG MUSCLE

(Two figures)

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WHEN one end of a striated muscle is injured, an electrical potential difference can be measured between injured and uninjured regions. This potential difference has been termed the injury potential. It is customary to regard this injury potential as due to the potential difference across the uninjured surface of the muscle fiber, the injured end merely serving as an indifferent lead to the inner protoplasm. Some evidence has been adduced to show that the injured surface plays a more important role in the production of the injury potential in invertebrate muscles (Steinbach, 1933); and Sugi (1934) has published figures showing that the magnitude of the injury potential of frog muscle can be altered by changing the concentration of the medium in contact with the injured surface.

Recent experimental work and theoretical considerations (Steinbach, 1935; Teorell, 1935; Meyer and Sievers, 1936) emphasize the role of all electrolyte phases in contact with membranes and particularly the protoplasmic electrolytes (Steinbach, 1935*a*). From our knowledge of the chemical constituents of muscle protoplasm it is possible to predict that diffusion potentials should arise between protoplasm and the usual biological environments and that these potential differences should account for a considerable portion of the measured injury potential, regardless of the nature of the membranes involved.

If diffusion tendencies of the protoplasm play an important role in the production of the injury potential, then diffusion potentials should arise at both injured and uninjured surfaces. In other words, the magnitude of the injury potential should be dependent upon the concentration of electrolytes in the solutions applied at either injured or uninjured surfaces. Sugi (1934) presents potential difference/log. concentration curves to show that this is, in general, true for frog muscle; but complete results are not given, and it is difficult to evaluate his findings. Since diffusion potentials have been shown to be of importance at both injured and uninjured surfaces in scallop muscles (Steinbach, 1933), it seemed essential to study further their role in frog muscle. Experiments relating to this are presented here.

METHODS

Sartorius muscles of common frogs (mostly *Rana pipiens*) were excised with as little injury as possible. In most cases they were immersed for about half an hour in Ringers fluid and then for another half-hour or hour in isotonic buffered sucrose solution. This latter step was for the purpose of washing out excess extracellular electrolytes—a procedure found to be essential for consistent results in the electrical measurements. The

sucrose solution was buffered with sodium phosphate mixtures, about 0.0005 M, at pH approximately 7.5. All salt solutions were similarly buffered.

In measuring injury potentials a fresh cut was made at the proximal end of the muscle, and the tissue then placed on a paraffin block so formed that the cut end could just make contact with fluid in one depression of the block, the uninjured surface being immersed in fluid in another depression (Fig. 1). A vaseline seal was placed between injured and uninjured surfaces of contact in such a fashion that only the cut surface was touched by the solution. Electrical contact to the solutions was made by saturated KCl bridges in contact with calomel half-cells. Care was taken to avoid contamination of the solutions by the bridges. Potential-difference measurements were made with a vacuum-tube voltmeter of high-input resistance.

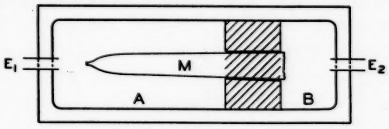

FIG. 1.—Diagram of arrangement of muscle on the paraffin block. The muscle (M) is situated so the cut end dips into solution B, while the uninjured end is immersed in solution A. The vaseline seal is shown by cross-hatching. E_1 and E_2 are salt bridges leading to the non-polarizable electrodes and voltmeter.

RESULTS

The system measured may be represented by the following chain with reference to Figure 1: electrode and bridge^a/ solution A^b/ uninjured muscle^c/ injured muscle^d/ solution B$^{a'}$/ electrode and bridge. The potential differences at a and a' are equal and opposite, hence the total measured potential difference will be due to other regions of the chain. The two interfaces that can be experimented with most easily are those represented as b and d—the uninjured and the injured surfaces. Asymmetry potentials might arise between injured and uninjured protoplasm as represented or perhaps due to the asymmetry of the membrane (b) itself (cf. Francis, 1937).

If the total injury potential is due, in the main, to diffusion tendencies of the protoplasmic electrolytes, then alteration of the salt concentration present in either fluid A or fluid B should have a considerable effect on the measured potential difference. Changes in potential difference as the salt concentration is altered will be termed "concentration potentials." Sugi (1934) has offered curves showing such concentration potentials, and his results find a ready explanation in terms of diffusion potentials. In my own work I have found it impossible to reproduce exactly the shape of the curves shown by Sugi, largely because of the fact that changes in potential difference due to concentration changes at the uninjured surface are only slowly reversible. Hence, the results reported here will not be offered as precise log. concentration/p.d. curves but will be concerned, in the main, with a comparison of the concentration potentials as salt concentrations at A and B are changed from 0.124 to 0.002 N or the reverse. Two salt solutions were used, KCl and NaCl. Dilutions were made with isotonic sucrose as described. Typical experiments are described in Figure 2. Representative values taken from such experiments are given in Table 1.

It is apparent that the magnitude of the injury potential is controlled by the concentration of KCl at the injured surface as well as at the uninjured. NaCl at the injured surface gives nearly the same concentration potential that KCl does, but a much lower one at the uninjured surface.

These results are invariable, provided that sufficient time is given for the equilibrium

values to be established (as much as half an hour or more for NaCl at the uninjured surface) and provided that the excess extracellular electrolytes are washed out before starting the experiment. That this last procedure should be important is not surprising,

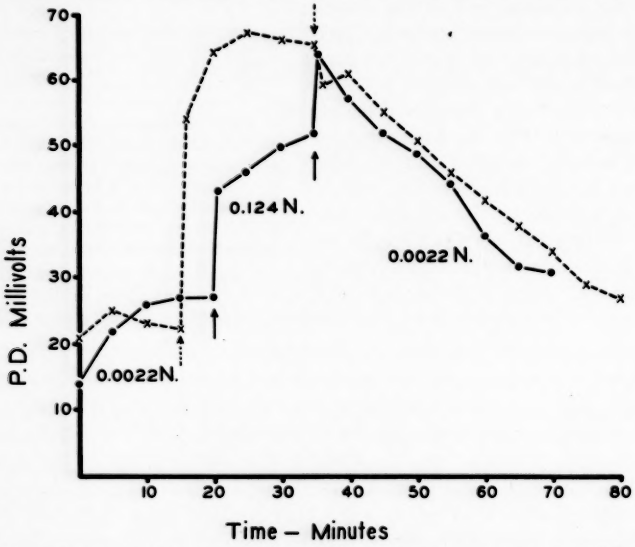

FIG. 2.—Typical experiments on two muscles showing concentration potentials (change in potential difference as the external concentration is changed) at the cut surface. In both cases the initial solutions were 0.0022 N in salt at both ends. Solutions changed at cut surface at the times indicated by arrows. - - - X - - -, KCl as salt. —— · ——, NaCl as salt.

TABLE 1*

CONCENTRATION POTENTIALS IN FROG SARTORII AS MEASURED BETWEEN THE SOLUTIONS INDICATED

FROG MUSCLE	SOLUTION	CONCENTRATION POTENTIAL (MILLIVOLTS)	
		Cut Surface	Uninjured Surface
1 (1	NaCl	+57	+18
1 (2	KCl	+54	+61
2 (1	NaCl	+51	+22
2 (2	KCl	+43	+56

* Concentrations used: 0.124 N and 0.0022 N. Dilute solution applied first in all cases. Plus signs refer to dilute solution.

since rather high diffusion potentials arise between NaCl solutions of different concentrations, and this factor added to the "short-circuiting effect" would greatly complicate the interpretation of the results.

The effects of salt concentration on injured and uninjured surfaces result, of course, in different injury potentials, depending on the composition of the leading-off fluids. This is shown by the figures in Table 2, which represent injury-potential measurements observed during the course of the same experiment given in Table 1. With isotonic NaCl at both ends of the muscle, a high injury potential is obtained. With dilute NaCl at both ends, the injury potential is much lower. With isotonic KCl at both ends, the injury potential is very low, and with dilute KCl is somewhat higher.

TABLE 2

INJURY POTENTIALS OF THE MUSCLES SHOWN IN TABLE 1 WITH EITHER CONCENTRATED OR DILUTE SOLUTIONS AT BOTH ENDS OF MUSCLE AS SHOWN

Frog	Muscle and Salt	Injury Potential (Millivolts)	
		0.002 N Both Ends	0.124 N Both Ends
1	1 NaCl	+ 9*	+48
	2 KCl	+14	+ 7
2	1 NaCl	+10	+39
	2 KCl	+21	+ 8

* Plus signs refer to uninjured surface as measured in the external circuit.

DISCUSSION

Several characteristics of the injury potential of frog muscle have been known for a long time. Among these may be mentioned the fact that potassium is particularly potent in reducing the value of the measured injury potential when applied to the uninjured surface. Less attention has been paid to the fact that the magnitude of the injury potential is controlled to a certain extent by the salt concentration of the external medium, regardless of the nature of the salt present. This latter point has been noted, however, several times and should be recognized. The results reported here add two other facts that must be taken into consideration in attempting to formulate the mechanisms giving rise to the injury potential (1) Changes in KCl concentration have essentially the same effects at either the injured or the uninjured surfaces. The end result of this is that there is little asymmetry shown between any two points on a muscle bathed in isotonic KCl. (2) Changes in NaCl concentration at the cut surface have essentially the same effect as changes in KCl concentration. Changes in NaCl at the uninjured surface give much lower concentration potentials. This, of course, has as an end result a rather high asymmetry potential with isotonic NaCl at both ends. An injury potential can be measured with Ringers fluid on both cut and uninjured surfaces.

The fact that KCl concentration has the same influence at either surface of the frog muscle suggests strongly that the surfaces become electrically identical under the influence of the high potassium, that is, an uncut surface treated with strong KCl becomes, in effect, an injured surface. This has been recognized experimentally for some

time. It is quite customary to produce an injury potential by treating one point on a cell or tissue with isotonic KCl.

On the other hand, the differences in NaCl concentration potentials show clearly that some definite asymmetry must exist in the system. Considering only a very simplified system in which unmodified diffusion potentials arise and in which concentration differences can be maintained by some mechanism, an easy explanation of all these results can be provided. Consider, for example, the following chain which in essence is like that described in Figure 1:

$$\underset{1.0}{\mathrm{NaCl}}/^{b}\ \underset{0.1}{\mathrm{K}X^{c}}/\ \underset{1.0}{\mathrm{K}X^{d}}/\ \underset{1.0}{\mathrm{NaCl}}$$

In this chain, concentrations are noted in relative units, and the NaCl solutions shown represent Ringers fluids at uninjured ($KX = 0.1$) and injured ($KX = 1.0$) surfaces. The membrane thus has only one-tenth the concentration of protoplasmic electrolytes present at the injured surface. If the mobility of the X anion is assumed to be 10, the mobilities of the other ions being as in water, the potential differences at the various interfaces can be calculated and from them the total measured potential difference of the chain. The chain shown above would have an "injury potential" of 51 millivolts (mv.) with the injured ($KX = 1.0$) surface negative. Dilute NaCl (0.02 relative concentration) at the two ends of the chain would give a total potential difference of only about 6 mv. Dilute KCl at the two ends would give a slightly higher result than with dilute NaCl. A concentrated KCl solution at either end would still give a total potential of about 40 mv., but if the membrane concentration is assumed to increase under the influence of the KCl the asymmetry vanishes, and the potential difference between the two ends vanishes. In general, then, such a simplified scheme accounts very well for the observed results if the assumptions are made that the membrane normally has a low electrolyte concentration and that electrolyte concentration increases upon injury (removal of the membrane) or upon KCl treatment.

It should be pointed out that this assumed increase in electrolyte content of the membrane is more strictly an increase in conductivity and could be due either to an actual increase in number of ions present or to an increase in mobility of ions in the membrane, provided the mobility ratios of the ions remained about the same. It seems unlikely that membranes specifically permeable to potassium alone would be operative, since such a membrane is obviously not present at the injured surface, and yet the KCl concentration potentials are the same at both surfaces. The possibility of a selective membrane is not ruled out by these observations, but it is rendered rather unnecessary if it is present. Another factor militating against the presence of a membrane selectively permeable to potassium is the fact that NaCl does have a definite concentration effect at the uninjured surface. It is unlikely that a surface could exist that allowed apparent penetration of NaCl and yet was highly selective for potassium.

The system that can account for the injury potential in terms of diffusion potentials between cellular and extracellular phases involves, as a necessary consequence, a cell surface that can maintain a low electrolyte concentration. Injury removes this layer, and apparently it is not regenerated to any great extent, at least during the times observed in these experiments. Possibly plug formation (see Heilbrunn, 1940) has something to do with this behavior.

Other interfaces than the ones considered in this simplified system are probably present and operative in giving rise to the injury potential. The fact that the injury potential is usually not quite zero when isotonic KCl is bathing both surfaces of the muscle is some indication of this. It might very well be true, for example, that injured protoplasm differs from normal protoplasm and that both are different in electrolyte makeup from the cell surface. If that is so, then there is at least one more interface present in the measured system which could be a source of asymmetry and can contribute to the injury potential.

SUMMARY

It is shown that concentration potentials are developed at both the injured and the uninjured surfaces of frog muscle with NaCl and KCl solutions. The concentration potentials with KCl at both surfaces and with NaCl at the injured surface are all of about the same magnitude and sign. NaCl concentration potentials at the uninjured surface are of the same sign but much lower.

These findings are discussed in terms of diffusion potentials, and it is shown that a simplified scheme postulating no selective membranes is adequate to explain the results.

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THE STIMULATING ACTION OF CITRATES AND OXALATES ON THE NEREIS EGG[1]

(Two figures and two plates)

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TREATMENT of the unfertilized egg of *Nereis limbata* with any one of several physical and chemical agents stimulates the egg to undergo a series of reactions, one of which is the breakdown of the germinal vesicle (Just, 1933; Heilbrunn and Wilbur, 1937; Wilbur, 1939). The process of germinal-vesicle breakdown has proved a convenient reaction for the study of stimulation and response in a response system consisting of a single cell. This response has been thought to be dependent upon the calcium of the egg cortex, for if the egg is first treated for a few minutes with an isotonic solution of sodium citrate, which presumably removes or binds at least a portion of the cortical calcium, the response to the stimulating agents is reversibly inhibited.

This inhibitory action of citrate is not peculiar to marine eggs. The use of citrates and oxalates in preventing the clotting of blood is well known. The following processes are also inhibited by citrates and oxalates or both: muscular contraction (Locke, 1894; Howell, 1894); heart beat (Koch, 1881; Howell and Eaton, 1893; Januschke, 1909; Gros, 1913; Salant and Hecht, 1915; McCartney and Ransom, 1917); intestinal contractions (Salant, Mitchell, and Schwartze, 1916; Salant and Schwartze, 1917); tone and amplitude of contraction of uterus (Sugimoto, 1913; Messini, 1929); tone of artery (Ellinger, 1926); rhythmic movements of stomach (Russo, 1935); vagus action on the heart (Shafer, 1936; Shafer and Crismon, 1936); and ciliary action (Gray, 1922; Wenner, 1931). On the other hand, citrates and oxalates do not always show an inhibitory action but may act as stimulating agents. This has been described for muscle (Locke, 1894; Garrey, 1905; Guenther, 1905; Rebello and Fontès, 1925); nerve (Loeb, 1901; Mathews, 1904; Moore, 1921; Fessard, 1936; Brink and Bronk, 1937; Brink, Sjostrand, and Bronk, 1939); cerebellum (Robertson and Burnett, 1912); heart beat (Russo, 1935); tone and amplitude of contraction of uterus (Sugimoto, 1913; Messini, 1929); contraction of muscles of blood vessels (Russo, 1935); intestinal contractions (MacCallum, 1903, 1904; Salant, Mitchell, and Schwartze, 1916; Salant and Schwartze, 1917; Salant, Kleitman, and Wright, 1922); and marine eggs (Loeb, 1902; Just, 1922). Clotting-time is decreased after injections of sodium citrate (Weil, 1915; Neuhof and Hirshfeld, 1922; Higgins and Fisher, 1924; De Souza and Hocking, 1934, 1935) and oxalic acid (Steinberg and Brown, 1939). Foster (1940) has not been able to confirm the results of Steinberg and Brown.

Now the question arises: If calcium is necessary for stimulation, as so many have thought, why should salts which bind calcium—such as citrates and oxalates—act as stimulating agents? The present experiments were carried out in an attempt to gain some insight into this stimulating action of citrates and oxalates and to decide, if possible,

[1] This paper constitutes a thesis presented to the faculty of the graduate school of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

whether or not calcium is also necessary for the stimulating action of citrates and oxalates or whether this type of stimulation is different from the other agents and can occur in the absence of calcium. If, in the *Nereis* egg, the stimulating action of citrates and oxalates should prove to be present after treatment of the egg with 0.35 M sodium citrate—a procedure which normally prevents stimulation—then calcium could no longer be considered a requisite for all types of stimulation. Information gained from a study of the *Nereis* egg as to the mechanism of the stimulating action of citrates and oxalates would presumably contribute to an understanding of the mode of action of these substances in the more complex systems mentioned above.

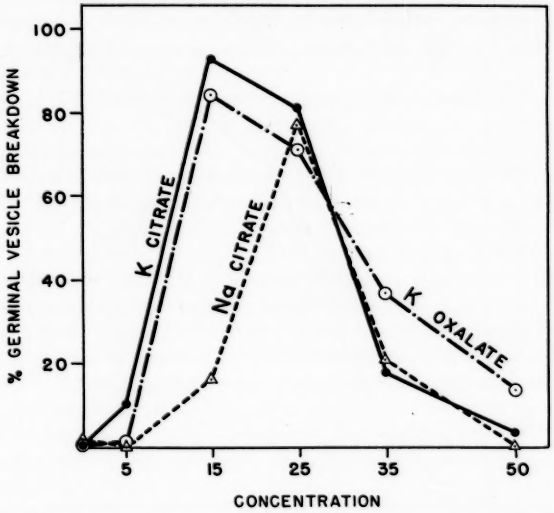

FIG. 12.—Each point represents the average value of 9–12 experiments. The concentration is expressed as parts of citrate or oxalate solution in 100 parts of a mixture of the citrate or oxalate solution and sea water.

METHODS

Eggs from a single female were washed once in sea water and concentrated in the center of a finger bowl containing approximately 250 cc. of sea water. One to 3 drops of eggs were then placed in each of a series of stender dishes containing from 30- to 40-cc. samples of the various concentrations of the substance under consideration. After a period of from 2 to 4 hours eggs were removed from each dish, compressed between a slide and cover slip, and the percentage of germinal-vesicle breakdown determined. At least 100 eggs were counted in each case, with one exception noted later. The points on the curves in Figures 12 and 13 represent the average values of 9 to 12 experiments.

The variation in percentage of germinal-vesicle breakdown between eggs from different females was considerable and is illustrated in the tables of data. In order that the samples counted might be representative, eggs were picked up from different parts of each dish. It is interesting to note that in certain solutions, at least, the eggs showing germinal-

vesicle breakdown were not distributed at random. For example, if one observes eggs in dishes containing mixtures of sodium citrate solution and sea water in which a considerable percentage, but not all, of the eggs have undergone germinal-vesicle breakdown, it is frequently seen that the eggs showing germinal-vesicle breakdown are localized in one or more areas. That is, in one region all or nearly all the eggs have undergone breakdown, while in another region nearly every egg may show an intact germinal vesicle. Dr. Josephine Hollingsworth[2] has observed this phenomenon in eggs activated by isotonic salt solutions.

In experiments in which eggs were transferred from one solution to another the amount of solution carried over never exceeded 3 drops. The room temperature at which the experiments were carried out varied between 21.6° and 26.3° C.

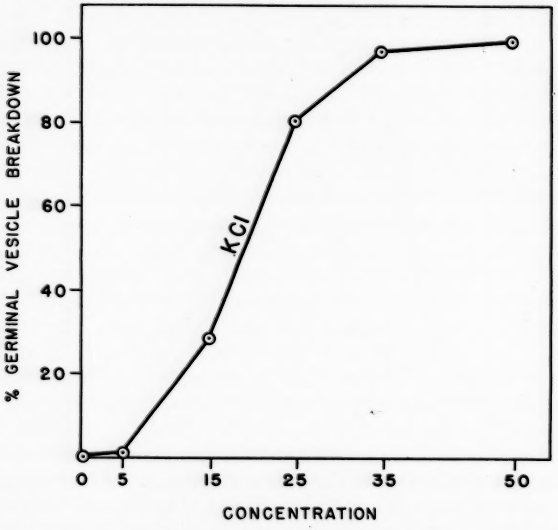

FIG. 13.—Each point represents the average value of 9 experiments

The following concentrations of solutions were employed: KCl, 0.53 M; NaCl, 0.53 M; potassium, sodium, and ammonium citrates, 0.35 M; and potassium and ammonium oxalates, 0.3 M. (Dr. F. Moser has found that a 0.35-M solution of sodium citrate causes neither shrinkage nor swelling of the *Arbacia* egg; and the concentration of citrates used in this study is based on this determination. The value of 0.3 M for the oxalate solutions may not be isotonic with the *Nereis* egg.) These solutions were mixed with sea water in various proportions, and the mixtures will be indicated as percentages of these solutions in sea water. For example, 25 parts of 0.53 M KCl added to 75 parts of sea water is termed a 25 per cent KCl–sea-water mixture. When citrates or oxalates are added to sea water, the osmotic pressure of the mixture will be somewhat different from that of the components taken individually, owing to the partial binding of the calcium and magnesium of the sea water.

[2] Personal communication.

Throughout the experiments attention was directed primarily to the phenomenon of germinal-vesicle breakdown. However, many interesting cortical and cytoplasmic changes resulting from treatment of the eggs with mixtures of citrates or oxalates with sea water were noted and will be briefly indicated from time to time but will not be discussed in detail.

POTASSIUM SALTS

Figure 12 indicates the percentage of germinal-vesicle breakdown in mixtures of sea water with potassium citrate and with potassium oxalate. The curves for potassium citrate–sea-water mixtures and potassium oxalate–sea-water mixtures are strikingly similar, both showing a high percentage of breakdown in the 15 per cent and the 25 per cent mixtures with much lower values for higher and lower concentrations. In a narrow range, then, mixtures of sea water with potassium citrate and with potassium oxalate have a pronounced stimulating action.

Some batches of eggs immersed in the potassium citrate–sea-water mixtures showed polar-body formation (Fig. 1). This was especially true of the 25 per cent mixture. In the 15 per cent and 25 per cent mixtures the oil drops moved to one side of the egg—a phenomenon which precedes cleavage after normal fertilization. Coalescence of oil drops was indicated by a decrease in number and an increase in the size of the drops. Membranes were present. Figure 2 shows an untreated *Nereis* egg. A small percentage of eggs were cytolyzed in certain mixtures. This was true not only of the potassium citrate mixtures but of the other citrates and oxalates as well.

In the potassium oxalate–sea-water mixtures polar-body formation was most common in the 15 per cent and 25 per cent mixtures but occurred occasionally in the 35 per cent and 50 per cent mixtures. Membranes lifted a considerable distance from the cell periphery were characteristic of the latter mixtures. This type of membrane is much different from that which follows fertilization. Often these membranes were double in character. (Fig. 3). (Cf. Costello and Young, 1939.) Abnormal cleavage is shown in Figures 4, 5, and 6.

Eggs were immersed in mixtures of isotonic KCl and sea water in order to determine whether the potassium ion, quite apart from citrate or oxalate action, was capable of initiating germinal-vesicle breakdown in concentrations of the same order of magnitude as used in the potassium oxalate– and potassium citrate–sea-water mixtures.[3] From the results summarized in Figure 13 it is evident that KCl is potent in producing germinal-vesicle breakdown. Polar-body formation and concentration of oil drops in one region of the egg took place (Fig. 7). In view of these results with KCl–sea-water mixtures the germinal-vesicle breakdown occurring in the potassium citrate– and potassium oxalate–sea-water mixtures cannot be definitely attributed to the effects of the citrate and oxalate ions but may have been due, in part at least, to the action of the potassium ion. However, the KCl mixtures differ from the citrate and oxalate mixtures in that a decrease in the percentage of breakdown does not occur with an increase in the concentration of KCl. A pure isotonic solution for KCl will also give a high percentage of breakdown (Heilbrunn and Wilbur, 1937).

SODIUM SALTS

Average percentages of germinal-vesicle breakdown in sodium citrate–sea-water mixtures are not greatly different from percentages obtained for the potassium citrate– and

[3] Although the percentage mixtures were the same for KCl as for potassium citrate and potassium oxalate, the concentration of the potassium ion is different in each case.

potassium oxalate–sea-water mixtures with the exception of a lower percentage for the 15 per cent sodium citrate mixture (Fig. 12).

Polar-body formation (Figs. 8 and 9), lobulation or budding-off of small portions (Fig. 10), and cleavage (Figs. 9 and 11) were characteristic of the 25 per cent mixture. Lobulation was also present in the 35 per cent mixture; and in certain batches some of the eggs cleaved in to several blastomeres (?) which were roughly similar in size. Coalescence of oil drops took place (Figs. 8, 9, and 11). A pigmented area was often prominent (Figs. 8, 9, and 11; see also Fig. 6).

Eggs immersed in NaCl–sea-water mixtures of the same concentration as those employed with KCl mixtures showed, with very few exceptions, little or no germinal-vesicle breakdown. This was true of eggs left in these mixtures 12 hours or longer. Nine batches of eggs were studied. The results indicate that the stimulating action of sodium citrate cannot be brought about solely by the sodium ion but that the stimulation must be related to the citrate ion.

AMMONIUM SALTS

Ammonium citrate and ammonium oxalate solutions mixed with sea water were much less effective than the other citrates and oxalates studied in producing germinal-vesicle breakdown. The concentrations used were the same as those employed for the previously mentioned citrates and oxalates. The highest percentage of germinal-vesicle breakdown was found in the 25 per cent ammonium oxalate mixtures, which showed an average of 25.3 per cent for 12 batches of eggs. Three batches showed high percentages of breakdown in certain concentrations. These particular mixtures had been prepared for 7 and 8 days before use. Freshly prepared solutions did not show this stimulating action. Both polar-body formation and cleavage were almost completely absent in the ammonium citrate and oxalate mixtures. However, the eggs from a single female did show abnormal cleavage in 25 per cent, 35 per cent, and 50 per cent ammonium oxalate mixtures. Single and double membranes were characteristic of the 50 per cent ammonium oxalate–sea-water mixtures.

Germinal-vesicle breakdown was almost completely absent in ammonium citrate mixtures. A "browning" of the eggs occurred in all concentrations of the ammonium citrate mixtures. In a 50 per cent mixture many or all of the eggs showed this at the end of 2 hours of immersion. Eggs in a 5 per cent mixture did not exhibit this brown coloration to any marked degree after 2 hours, but here, too, many or all became brown a few hours later.

INHIBITORY ACTION OF CITRATES

Previous work had demonstrated that a few minutes' treatment of the *Nereis* egg with 0.35 M sodium citrate would reversibly inhibit the stimulating action of isotonic NaCl, KCl, and ultra-violet irradiation (Heilbrunn and Wilbur, 1937). This suggested an interesting experiment. The data cited above show that citrate–sea-water mixtures act like other stimulating agents in producing germinal-vesicle breakdown. If this stimulating action of the citrate–sea-water mixtures is comparable to the stimulating action of the NaCl, KCl, and ultra-violet irradiation, then a pretreatment with 0.35 M sodium citrate should also inhibit the stimulating action of the citrate–sea-water mixtures. The experiment has been carried out, and it has been found that the 0.35 M sodium citrate does indeed reversibly inhibit the stimulating action of the sodium citrate–sea-water mixtures. Table 1 gives the results of such experiments in which eggs were immersed in

0.35 M sodium citrate for 6 or 10 minutes and then transferred to 15 per cent and 25 per cent sodium citrate–sea-water mixtures. The inhibition of breakdown in 25 per cent

TABLE 1

INHIBITORY ACTION OF 0.35 M SODIUM CITRATE

No. of Expt.	Immersion Time in 0.35 M Sodium Citrate (Minutes)	Percentage Breakdown in a 15 Per Cent Sodium Citrate–Sea-Water Mixture	Percentage Breakdown in a 15 Per Cent Sodium Citrate–Sea-Water Mixture Following Citrate Treatment	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture Following Citrate Treatment
1	6	97	21	98	0
2	6	2	52	92	16
3	6	98	16	99	0
4	6	94	10	96	8
5	6	11	0	99	21
6	10	52	40	98	10
7	10	95	40	98	12
8	10	29	19	91	0
9	10	5	16	60	6
10	10	73	1	84	8
11	10	59	78	94	10
12	10	7	11	66	18
13	10	80	67	97	18
14	10	86	42	98	1
15	10	52	51	88	6

TABLE 2

INHIBITORY ACTION OF 0.35 M POTASSIUM CITRATE

No. of Expt.	Immersion Time in 0.35 M Potassium Citrate (Minutes)	Percentage Breakdown in a 15 Per Cent Potassium Citrate–Sea-Water Mixture	Percentage Breakdown in a 15 Per Cent Potassium Citrate–Sea-Water Mixture Following Citrate Treatment	Percentage Breakdown in a 25 Per Cent Potassium Citrate–Sea-Water Mixture	Percentage Breakdown in a 25 Per Cent Potassium Citrate–Sea-Water Mixture Following Citrate Treatment
1	6	.....	0	.....	0
2	6	.....	0	.....	0
3	6	.....	0	.....	0
4	6	100	0	1	0
5	6	100	0	86	2
6	6	100	0	97	0
7	6	94	1	84	0
8	6	99	0	57	0
9	6	100	0	35	0

mixtures following treatment with 0.35 M sodium citrate is especially marked (last two columns of table). Experiments 2 and 11 provide exceptions to the inhibition in the 15

per cent mixture. Here it is seen that germinal-vesicle breakdown is greater in eggs treated with 0.35 M sodium citrate before immersion in the 15 per cent mixture than in untreated eggs placed in the same solution. These will be discussed later. Experiments 9 and 12 appear to be similar, although the increased breakdown following treatment with 0.35 M citrate may be within the experimental error of counting. This

TABLE 3

REVERSIBILITY OF THE INHIBITORY ACTION OF 0.35 M SODIUM CITRATE

No. of Expt.	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture Following 10 Min. Treatment with 0.35 M Sodium Citrate	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture Following Immersion in 0.35 M Sodium Citrate, 25 Per Cent Sodium Citrate–Sea-Water Mixture, and Sea Water	Remarks
1	94	64	76	Eggs immersed in 25 per cent sodium citrate–sea-water mixture for 1 hour, 15 minutes; then transferred to 30–40 cc. of sea water for 15 minutes
2	100	9	91	Same
3	100	21	60	Same
4	99	32	45	Same
5	96	8	8	Eggs immersed in 25 per cent sodium citrate–sea-water mixture for 20 minutes. Other conditions unchanged
6	98	8	46	Same
7	95	2	37	Same
8	90	0	87	Eggs immersed in 25 per cent sodium citrate–sea-water mixture for 20 minutes; then transferred to 200–250 cc. of sea water for 30 minutes
9	33	2	31	Same
10	90	33	89	Same
11	75	5	68	Same
12	98	59	81	Same
13	87	41	85	Same

behavior was never encountered in the 25 per cent mixture of sodium citrate or in either the 15 per cent or the 25 per cent mixtures of potassium citrate (Table 2). Six minutes' immersion in 0.35 M potassium citrate inhibited the stimulating action of 15 per cent and 25 per cent mixtures of potassium oxalate.

The eggs which have been inhibited by the 0.35-M citrate are still capable of responding to stimulation after the citrate has been removed, indicating that the 0.35-M solution did not inhibit the reaction by injuring or killing the eggs. The eggs were first treated as previously described; that is, they were placed in 0.35 M sodium citrate followed by the

25 per cent sodium citrate–sea-water mixture. They were then put into sea water to remove the citrate and again placed in the 25 per cent mixture. Table 3 shows that the inhibitory action of the 0.35 M is reversible, for the eggs respond to the final 25 per cent sodium citrate mixture after immersion in sea water. The conditions under which the last 5 experiments were carried out gave almost complete reversibility (see last column of Table 3).

Similar experiments showed the inhibitory action of potassium citrate to be reversible. A 15 per cent mixture was used. Since only 3 drops of eggs were used in the first solution, the loss of eggs in successive transfers did not always permit the counting of 100 eggs in the final solution of potassium citrate and sea water.

Germinal-vesicle breakdown in 15 per cent potassium oxalate is also inhibited if the eggs are first immersed in 0.3 M potassium oxalate for 3–6 minutes. However, the 0.3 M potassium oxalate is unsatisfactory in certain respects. For example, some of the eggs become very dark after a few minutes in this solution, making the determination of the presence of a germinal vesicle difficult and uncertain. An attempt was made to ascertain whether or not the inhibitory effect of 0.3 M potassium oxalate was reversible. The procedure was similar to that described for sodium and potassium citrate. In 5 series carried out there was no indication that the inhibitory action of potassium oxalate is reversible. In 2 series all the eggs were either very dark or completely ruptured in the final solution.

DISCUSSION

The results have shown that the *Nereis* egg, like several other systems mentioned above, is stimulated by citrates and oxalates. In appropriate concentrations in sea water certain citrates and oxalates cause the *Nereis* egg to undergo germinal-vesicle breakdown and to give off polar bodies. Isotonic NaCl and KCl solutions and ultra-violet irradiation also exert a stimulating action on this cell. To suppose that the same series of reactions is initiated by all these stimulating agents would seem more reasonable than to postulate a different mechanism for each agent. And, indeed, these agents are similar in that the stimulating action of each is reversibly inhibited if the eggs are first treated briefly with 0.35 M citrate.[4] In view of this inhibiting action of a calcium-binding compound, it is tempting to believe that calcium is concerned whenever the egg is stimulated. This point of view has been stressed by Pasteels (1935, 1938).

The suggestion has been made (Heilbrunn and Wilbur, 1937; Wilbur, 1939) that a stimulating agent acting upon the egg upsets the normal equilibrium of the egg in one way or another and calcium is released from a calcium-proteinate compound in the egg cortex. The free calcium may then initiate the series of changes which lead to germinal-vesicle breakdown and other maturation processes. The same hypothesis may also be applied to the stimulating action of the mixtures of sea water and citrate or oxalate. Without attempting to specify the changes which these solutions might produce, one may suppose that the normal equilibrium of the egg is altered by these mixtures and that calcium is available to the extent that the processes of germinal-vesicle breakdown, polar-body formation, etc., can take place. The inhibitory action of the stronger concentrations of citrate on such a scheme would be due to a more rapid or more complete removal of calcium with a consequent absence of stimulation. Guenther (1904) believed

[4] The reversibility of the action of 0.35 M potassium citrate in inhibiting a potassium oxalate–sea-water mixture has not been tested.

that the stimulating action of sodium oxalate on muscle was in some way dependent upon the presence of the calcium ion. Loeb (1901) proposed that citrate by removing calcium from the outer layers of a muscle fiber increased the irritability. As calcium diffused outward and replaced that removed by citrate, the fiber became less irritable. Heilbrunn (1940) thinks that citrates and oxalates may remove magnesium from the cell faster than calcium and that the upset in the normal calcium-magnesium ratio which results produces stimulation.

It will be remembered that treatment of eggs with 0.35 M sodium citrate does not always inhibit germinal-vesicle breakdown on transfer to a 15 per cent sodium citrate–sea-water mixture. On the other hand, 0.35 M potassium citrate does prevent the action of a 15 per cent potassium citrate–sea-water mixture. The explanation of this difference seems to be indicated in the experiments with mixtures of sea water with NaCl and KCl in which it was found that the sodium ion is much less potent than the potassium ion in initiating germinal-vesicle breakdown. In terms of the hypothesis suggested above, this would mean that the sodium ion is less effective than the potassium ion in releasing bound calcium. If the sodium ion is not able to release as much calcium, then, perhaps, sodium citrate will remove or bind less calcium than potassium citrate; or, in other words, after treatment with sodium citrate an egg will contain more available calcium than after treatment with potassium citrate. And if sufficient calcium is available, then treatment with a citrate–sea-water mixture will result in stimulation.

From the present discussion it is evident that at present no complete explanation of the stimulating action of citrates and oxalates can be given. The point of view presented here has been that citrates and oxalates, like other stimulating agents, in some manner bring about an increase in free calcium within the cell. The fact that the stimulating action of citrate and oxalate is inhibited when the cell is first immersed for a few minutes in a pure solution of citrate indicates that a sufficient concentration of calcium must be present in the cell even for a response to these calcium-binding compounds, and, accordingly, the stimulating action of citrates and oxalates is not inconsistent with the view that calcium is always necessary for the response of the *Nereis* egg to stimulation.

SUMMARY

1. Solutions of sodium citrate, potassium citrate, or potassium oxalate mixed with sea water in certain proportions stimulate the *Nereis* egg to undergo germinal-vesicle breakdown, polar-body formation, and other changes.

2. The response of the egg to stimulating concentrations of mixtures of sodium or potassium citrate and sea water can be reversibly inhibited by first treating the eggs for a few minutes with 0.35 M sodium or potassium citrate. The inhibitory action of 0.3 M potassium oxalate was not found to be reversible.

3. Solutions of ammonium citrate or ammonium oxalate mixed with sea water have little stimulating action. Ammonium citrate–sea-water mixtures produce a "browning" of the eggs.

4. Mixtures of isotonic KCl and sea water cause germinal-vesicle breakdown and polar-body formation.

5. Mixtures of isotonic NaCl and sea water are much less effective than KCl–sea-water mixtures.

This work was carried out under the direction of Doctor L. V. Heilbrunn. I am indebted to him for his kind suggestions concerning the preparation of the manuscript, to Doctor C. A. Angerer for helpful discussion of the problem, and to Mr. L. Santamaria for translation of an Italian reference. I gratefully acknowledge a grant from the University of Pennsylvania chapter of Sigma Xi.

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PLATE I

FIG. 1.—15 per cent potassium citrate–sea-water mixture. Two polar bodies are seen beneath membrane.

FIG. 2.—Untreated egg in sea water. Intact germinal vesicle is indistinctly shown in center.

FIG. 3.—50 per cent potassium oxalate–sea-water mixture. Intact germinal vesicle is seen in the center of egg. Note membrane.

FIG. 4.—35 per cent potassium oxalate–sea-water mixture. Abnormal cleavage has taken place in some eggs.

FIG. 5.—25 per cent potassium oxalate–sea-water mixture. Abnormal cleavage is shown.

FIG. 6.—15 per cent potassium oxalate–sea-water mixture.

PLATE I

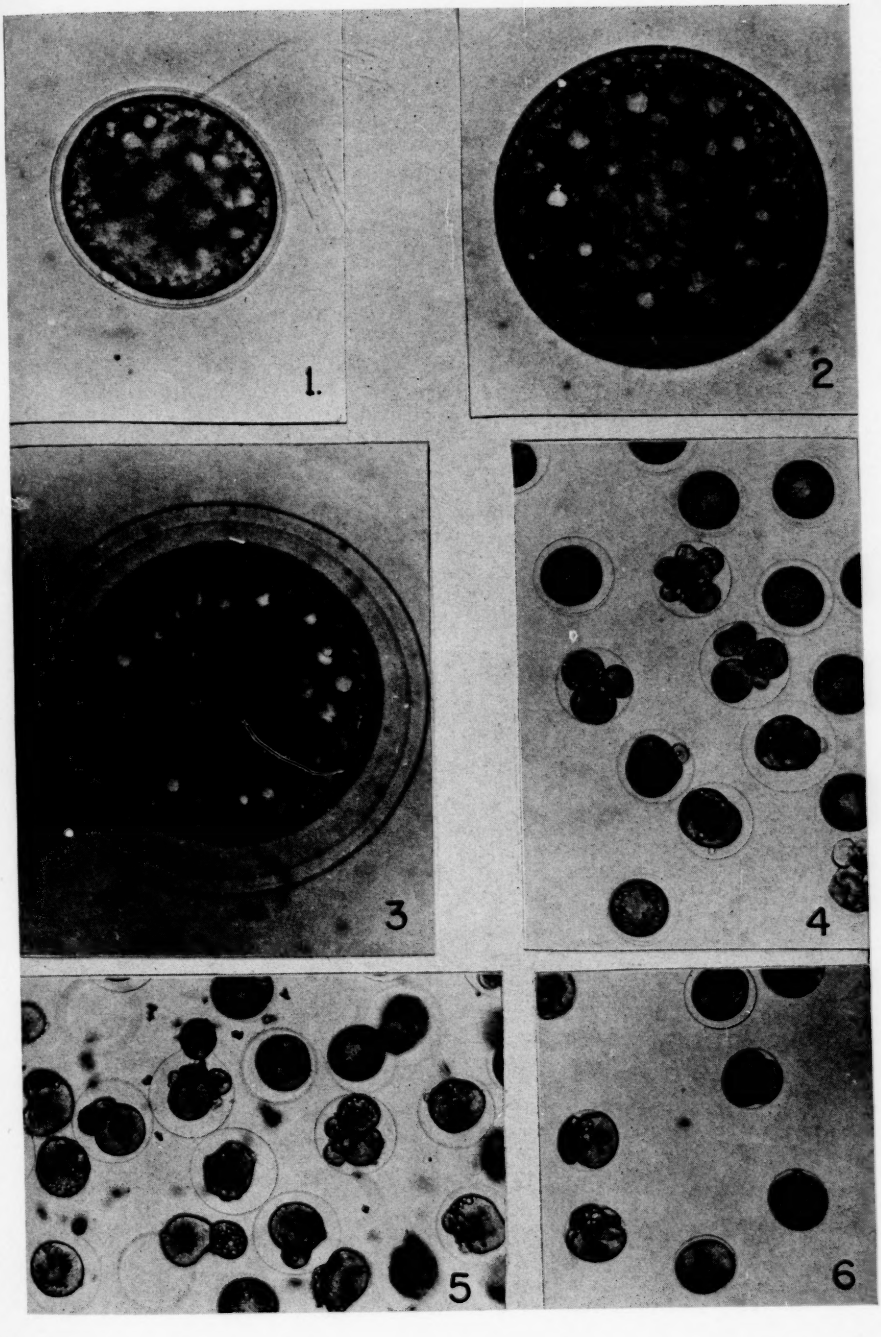

PLATE II

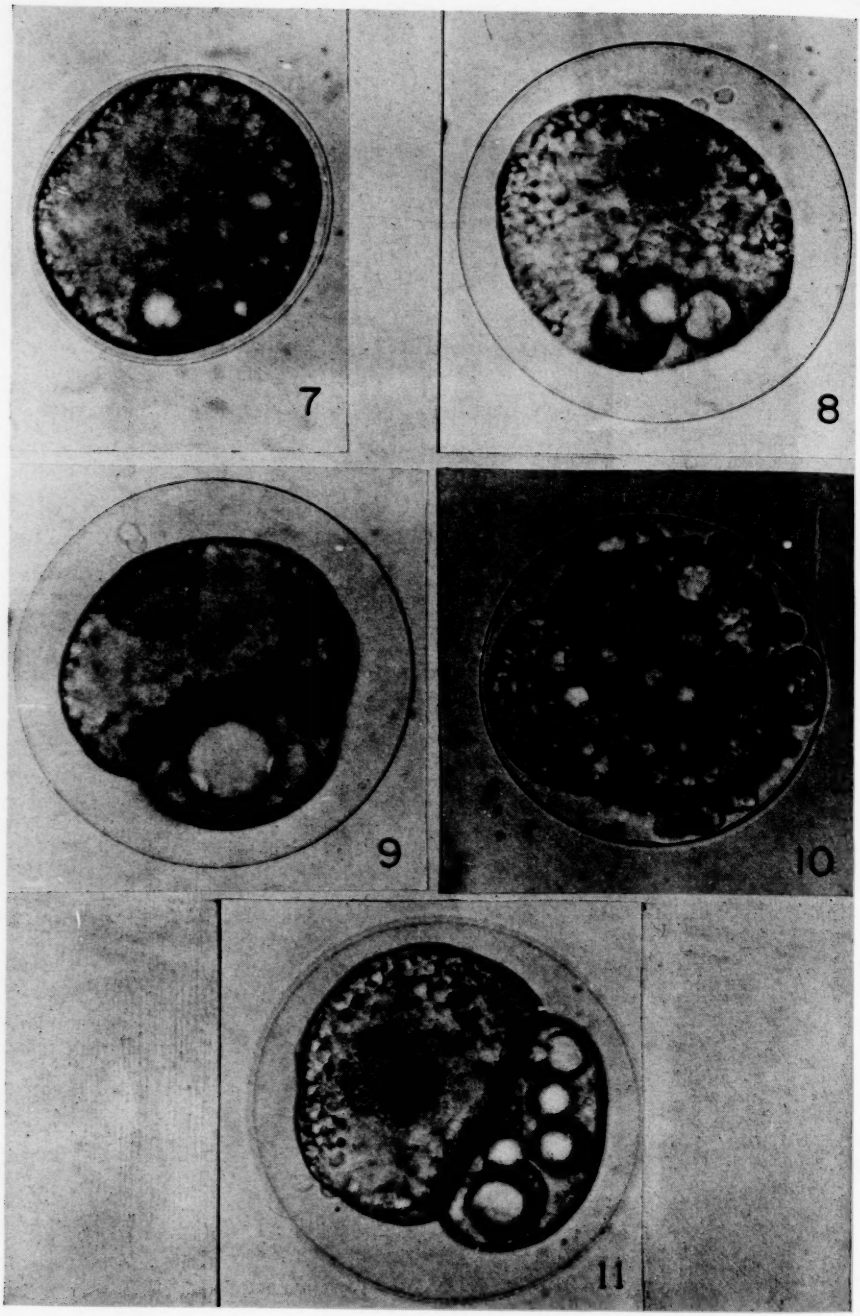

PLATE II

FIG. 7.—50 per cent KCl–sea-water mixture. Two polar bodies are present beneath membrane. Oil drops have moved to one side of the egg.

FIG. 8.—25 per cent sodium citrate–sea-water mixture. Two polar bodies are shown. Oil drops have coalesced.

FIG. 9.—25 per cent sodium citrate–sea-water mixture. Cleavage has taken place. Oil drops have coalesced forming 3 large drops. Two polar bodies have been given off. Pigmented area is seen near polar bodies.

FIG. 10.—25 per cent sodium citrate–sea-water mixture. Small portions of the egg have been budded off.

FIG. 11.—25 per cent sodium citrate–sea-water mixture. Cleavage has occurred. Coalescence of some of the original oil drops has occurred. Polar bodies are seen at the surface of larger blastomere. Pigment is concentrated in center of larger blastomere.

THE MELANOPHORE RESPONSES OF FISHES IN RELATION TO CONTRAST IN THE VISUAL FIELD[1]

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HOW does a fish adjust the shade of its integument to correspond with the background on which it is placed? It was in an attempt at a partial answer to this question that Sumner (1911) adapted to fish the "ratio hypothesis" of Keeble and Gamble (1904) which had been originally proposed to explain changes of shade in certain crustacea. According to this hypothesis, the factor which determines and makes possible the appropriate degree of integumentary pigment dispersion is the ratio of direct to reflected light which reaches the eyes of the animals, that is, the ratio between the light coming from above and the light reflected from surrounding objects. The very large value of this ratio on a black background produces maximal pigment dispersion; the very small value of the ratio on a white background results in maximal pigment concentration; and the particular value of the ratio on any background of intermediate shade determines the particular degree of pigment dispersion on that background. In other words, the greater the value of the ratio, the greater is the dispersion of pigment, and the darker is the shade of the fish.

Attempts to test this hypothesis have frequently yielded somewhat uncertain results. Sumner himself (1911) tried to obtain an abnormally low ratio of light above to light below by covering the vessels containing turbots and admitting light from below only but found that instead of becoming pale, as expected, most of the fish became somewhat dark. He believed that unwanted reflections played too large a part in his experiments. Mast (1914) from work with flounders concluded that "the interaction of the light received from the different immediate sources is probably not so simple as is demanded by the ratio hypothesis of Keeble and Gamble." Sumner and Keys (1929) made tests with two backgrounds on which the illumination was such that the ratio was in one case greater, in the other less, than that which would ordinarily exist. They found that the responses of *Hypsopsetta guttulata* agreed with the ratio idea but decided that "ratio" ought not to be quantitatively construed. Pearson (1930), with *Ameiurus*, repeated the work of Sumner and Keys and obtained similar results, but under other experimental conditions he did not find responses to be at all in conformity with the hypothesis. However, Brown (1936) measured the ratios existing on a series of black, gray, and white backgrounds and found that at intensities of 1.75 foot-candles or more the diameter of the melanin masses of *Ericymba buccata* varied directly with the ratio.

But, even though the truth of the hypothesis be granted, there may be a question as to how the ratio produces its effect. It may be that the response of the fish is a response to contrast as such; that the fish "sees" a contrast much as man does and makes adjustments accordingly. It may be, on the other hand, that the relationship between receptor and effector is a more direct one; that stimulation of one part of the retina leads directly

[1] I am grateful to Dr. D. E. Minnich of the University of Minnesota for many favors received both while this work was in progress and at other times.

to expansion, of another to contraction, of the melanophores. If so, the exact state of the melanophores will depend on the extent to which one or the other of these opposing stimuli predominates.

Investigators do seem to agree that adjustments of shade in fishes are made according to the relative stimulation of the upper and lower parts of the retina. Under natural conditions, of course, this would inevitably result from the position of the lighter sky above and the darker background below, but it appears that there may be a corresponding dorsoventral polarization and localization of function in the fish eye itself. Von Frisch (1911) found that covering either the entire eyes or the lower halves of the eyes of trout led to darkening but that covering the upper halves was without appreciable effect on changes of shade. Effects were, however, much greater when the lower halves only were covered than when the whole eyes were covered, and this was interpreted to mean that light reaching the upper halves was involved in the production of a contrast effect. His method of investigation was not entirely satisfactory, since the black paste used to cover the eyes frequently came loose or fell off in a very short time. An improved technique of the same type, consisting of the use of false corneas, or "goggles," was used by Sumner (1933). When the lower half of the visual field of *Fundulus parvipinnis* was darkened by this method, the fish became dark regardless of background. When the upper half was darkened, no visible change usually occurred on white, though a slight darkening might occur; but greater pallor than normal was seen when the fish were on a dark background. Darkening other parts of the visual field failed to give clear results, and attempts to rotate the eye in its socket without interfering with its function were unsuccessful. Sumner concluded from these experiments that the shade assumed by the fish was determined by the relative luminosity of the upper and lower parts of the visual field. Finally, Butcher (1938) found that

> covering the eye, rotating the eye, rotating the fish, destroying either the lower or upper region of the eye, and illumination from above or below on different backgrounds, all definitely show that the upper region of the retina of *Fundulus* is related to the pale response and the lower portion to the darkening of the body.

Nevertheless, I doubt that changes of shade in fishes can be wholly explained on the basis of the polarity of the eye. It is worthy of note that both in attempts to reduce the ratio of "direct/reflected" illumination to abnormally low values and in experiments which involved partial covering of the eyes a theoretically unexpected darkening of the fish has frequently appeared. Such darkening has commonly been interpreted, according to the nature of the experiment, as an effect of stray light reaching the eye by reflection or otherwise, or of light reaching receptors through the tissues of the head. In the following experiments with abnormally low ratios such darkening has been so marked that another explanation has seemed necessary.

EXPERIMENTS AND RESULTS

A number of experiments with an abnormally high proportion of light coming from below, similar to those originally performed by Sumner, were first made. A black wooden box, of dimensions to fit inside a small glass aquarium, 13 × 26 × 10 cm., was simply placed inside the aquarium containing the fish so as to shut off all light except from the bottom. To reduce the possibility of reflections at the upper surface of the water an opening in the top of the box, which could be closed by a blackened cork, allowed the

escape of air; and the box was weighted so that no air space would remain above the water. Light of 10 foot-candles intensity, measured with a Weston illuminometer, was admitted through the bottom of the aquarium. Without appreciable difference in the results the light was sometimes transmitted through a sheet of opal glass under which a lamp was placed, sometimes reflected from a white enamel basin or from a sheet of white cardboard.

Under these conditions 46 individual tests, each preceded by adaptation for an hour to a black background under an illumination of 10 foot-candles, were made with a total of 15 specimens of the freshwater minnow, *Nocomis biguttatus* Kirtland. It was found that none of the fish remained maximally dark under the black box, but, on the other hand, none became pale. The fish in 15 tests were recorded as "slightly dark," indicating a considerable, but by no means maximal, blanching; in 31 tests as "moderately dark." If a low ratio of light in the upper part of the visual field to light in the lower part always produced contraction of the melanophores, these fish might have been expected to turn pale even more readily than on an ordinary white background.

In further tests the apparatus was changed, chiefly in order to provide greater ease of handling and observation. The darkened area in the visual field of the fish was somewhat reduced. The animals were placed in an 18-cm. crystallizing dish fitted with a floating black wooden lid, again provided with an opening for the escape of air. The depth of water used was just enough to allow the fish freedom of movement; however, they seldom moved except when first put into the dish. The dish rested on a glass plate, 75 × 75 cm., below which was supported a sheet of white cardboard. This apparatus was surrounded by sheets of opal glass, outside of which lamps were arranged on a level with the space between the cardboard and the glass plate. Adjustments were again made so that the intensity of the light reaching the bottom of the dish was about 10 foot-candles. This arrangement produced a "bottom" which appeared more clearly white than any that could be obtained with light transmitted directly from below through any available diffusing screen. Essentially, the fish were on a white background with a black area immediately above them.

Six of the fish which had previously been tested under the black box were adapted to a black background and tested again with the new equipment. Again, all remained somewhat dark. In each of 2 trials, 3 of the fish were found to become "slightly dark," while the other 3 were considered "moderately dark." As far as subjective impressions go, pigment dispersion appeared to be very little less than it had been when both the top and the sides of the container were dark. Removal of the black lid and the addition of light of about 20 foot-candles intensity from above quickly brought about a pronounced, persistent pallor.

The same 6 fish were also tested after being thoroughly blanched by adaptation to a white background for an hour. Under the black lid all 6 darkened slightly but definitely in each of 2 tests. Twenty-eight other fish taken at random were likewise individually adapted to a white background and then left under the black lid for ½ hour. Of these, 3 remained very pale, although there was some doubt as to whether they remained maximally so, 8 showed a very definite slight darkening, 14 became moderately dark, and 3 were considered "dark." It was quite clear that with the upper part of the visual field dark and the lower part light, most of these pale fish became darker.

The next step—again with the same 6 fish that had been carried through the preceding experimental variations—was to leave the apparatus as before except that the black

lid was not used. Light then reached the fish from above by reflection from the white walls and ceiling of the room, the intensity of such light reaching the dish being 2 foot-candles. The light strength from below remained at 10 foot-candles. Under these conditions in each of 2 trials after previous adaptation to a black background 3 of the fish assumed a shade approaching an intermediate one, 2 blanched until they were only slightly dark, and 1 became very pale. The tendency toward melanophore contraction appeared to be somewhat greater than when the black cover was used, but except for the one fish it was still clearly incomplete. On the other hand, when the same fish were exposed to light of 2 foot-candles from above only, in the same dish, with the same cardboard under the glass as a background, approximately complete pallor appeared to result in all cases.

When the fish were first adapted to a white background and then exposed to 2 foot-candles illumination from above and 10 from below, again in each of 2 trials 1 darkened moderately, 4 darkened slightly, and 1 darkened slightly in one test but remained very pale in the other. No such darkening occurred when, over a white background, the fish were exposed to light of 2 foot-candles intensity from above only.

As a final and more critical test, when a shortage of *Nocomis* developed, 12 creek chubs (*Semotilus atromaculatus* Mitchill) were individually exposed to an illumination of 2 foot-candles intensity from above and 10 from below, with the same equipment as before. After 1 hour in this situation the fish were killed by being thrown into hot fixing fluid, and part of the integument was removed, mounted in glycerin, and examined. In each case a control animal, selected as closely as possible for similarity in size, appearance, and reaction to background, was used. This animal was immediately before or immediately afterward exposed to light of 2 foot-candles intensity from above only, in the same dish with the same cardboard as a background under the glass plate on which the dish rested. After an hour this animal was also killed, a piece of integument from the same part of the body was similarly prepared, and the two were compared under the microscope.

Strictly quantitative comparisons were not attempted, but the slides were examined not only by the experimenter but also by at least one unprejudiced observer, who without other knowledge of the experiment was asked to judge whether there was any difference in the condition of the melanophores on the two slides. In every case the melanophores were found to be more expanded in the fish which in addition to the light from above had received stronger illumination from below. Moreover, not only was pigment dispersion in a given individual greater than in its individual control, it was greater than in any control.

With few and somewhat doubtful exceptions, therefore, a partly expanded condition of the melanophores resulted whenever light reached the fish more strongly from below. Pigment dispersion appeared to be only slightly less when the top of the container only was darkened than when both the top and the sides were darkened; and some dispersion clearly occurred even when, in addition to the light from below, a weaker light was allowed to reach the fish from above.

DISCUSSION

It is clear that the total light intensity used in these experiments was more than sufficient to bring about or to maintain contraction of the melanophores. In some of the

control experiments mentioned a much lower total intensity did bring about such contraction. Nor does it seem probable that the total intensity was too great to allow normal responses, for it was far less, for example, than that which would be returned to the eyes by a white background in bright daylight, where complete melanophore contraction occurs.

That undesired reflections played any considerable part in these experiments also appears improbable. No position could be found from which to the observer's eye the bottom of the vessel containing the fish appeared dark or the lower side of the cover appeared light. Moreover, no change in the behavior of fish exposed to light from above in either black or white dishes could be detected when the dishes were covered by one or more sheets of glass, even when the glass was not in contact with the water. With a diffuse-light source above, a fish in a black-bottomed glass dish covered by glass touching the water at every point must be in a situation very much like the one under the black cover as far as the incoming light and its reflections are concerned, except that the positions of the dark and light fields are interchanged. It would seem, therefore, that reflections can be ruled out and that the only significant effect of the black cover was to exclude light from above.

It has thus been shown that over a background on which melanophore contraction occurs under ordinary conditions a small ratio of light from above to light from below does not necessarily induce such contraction; that a very low ratio, with most of the light coming from below, may even bring about a partial melanophore expansion.

Evidently, therefore, a certain definite value of the ratio of "direct/reflected" illumination is required to bring about complete contraction of the melanophores. It might be suggested, then, that when the value of the ratio is very low, no background response occurs, just as none occurs when optic stimulation is completely absent. As a matter of fact, tests with 30 *Semotilus* did show that their appearance after an hour in the dark was very similar to their appearance after an hour under the black lid. The results in the dark were: 6 "pale," 12 "slightly dark," and 12 "moderately dark." Under the black cover the corresponding figures were: 5 "pale," 12 "slightly dark," and 13 "moderately dark." None were either maximally pale or maximally dark in either situation.

I believe that this signifies, not that the two situations are identical, but merely that the total activation of the melanophores is just enough to bring about a partly expanded condition in either case. In the dark, light forms no part of the total stimulation; in the light, the effects of light normally dominate all others. The difference between the two situations becomes clear from a consideration of the time required for melanophore change. In these particular trials there was no way of determining the exact time required for change in the dark. In other tests which have been made, however (cf. Danielson, 1938), the minimum time required to approach the equilibrium condition in darkness seemed to be at least ½ hour. Five or 10 minutes, as a rule, brought little or no change. The changes under the black cover, on the other hand, appeared to be completed in not more than 5 or 10 minutes. The two situations are, therefore, quite different, even though they result in about the same final appearance of the fish.

If we compare the foregoing experimental results from the use of very low ratios of "direct/reflected" light intensity—or, more properly, intensity above/intensity below—with the known results over various normally illuminated backgrounds, certain possibilities appear. Under an illumination of 10 foot-candles, a condition of 10/0 is normally approached, though it is not experimentally reached, over a black background. In the

experiments just considered, the reciprocal of this, namely, 0/10, must have been approached when the fish were under the black covers. The result in the first instance is complete expansion of the melanophores; in the second, considerable, though incomplete, expansion. A ratio of 10/2 would be found over some gray background, while the ratio of 2/10 was established in one set of experiments. The condition of the melanophores over a gray background is one of partial expansion; and partial expansion, to a degree somewhat less than that obtained with a ratio of 0/10, also resulted when a ratio of 2/10 was used. The ratio reaching the fish eye over a white background illuminated from above could not be measured exactly because of shading by the instruments used, but it appeared to be between 10/6 and 10/8. There is certainly less difference between the numerator and the denominator over a normally illuminated white background than in any of the ratios previously mentioned. The effect of an ordinary white background is complete contraction (or no expansion) of the melanophores. The effect of either the ratio 0/10 or 2/10, therefore, resembles that of its reciprocal more than it resembles the effect of a normally illuminated white background. Moreover, melanophore expansion is greater with a ratio of 10/0 than with a ratio of 10/2; and in the same way, though perhaps less obviously, melanophore expansion is greater with a ratio of 0/10 than with a ratio of 2/10. The conclusion suggests itself that the ratio works either way, that the situation and the response are essentially similar whether the ratio is a/b or b/a.

It is true that the response to a situation in which more light comes from below differs in degree from the response to the more usual situation in which more light comes from above. Expansion of the melanophores is less under a black cover than over a black bottom. It is evident that the different parts of the retina are not completely interchangeable in the reception of the differences in illumination that form the stimuli for changes in shade; that a considerable specialization has occurred, either in different parts of the eye or in central nervous connections or both. It has been shown by Wunder in several papers (Wunder, 1933) and by Butcher (1938) that marked differences in the distribution of rods, cones, and retinal pigment between the upper and the lower parts of the fish retina commonly exist. It may be that because of such structural differences the fish "sees" a less vivid contrast when the conditions of illumination are the inverse of those naturally occurring. Specialization with respect to central connections is, of course, equally possible.

The fact remains that expansion of the melanophores in *Nocomis* and *Semotilus* occurs when the conditions of illumination are the inverse of those which normally evoke that response. Moreover, the same response to this situation, though unrecognized as such, seems to have been observed in other fishes. As previously mentioned, more or less melanophore expansion has frequently been described as a result of experiments involving either abnormal increases in the illumination from below or covering the upper part of the eye. All this indicates that the fish eye is much less strongly polarized than has been generally assumed. Although all parts of the eye are not equivalent, the state of the melanophores appears to be determined not by the stimulation of particular regions of the retina but by the degree of contrast in the visual field as a whole.

SUMMARY

Various partly expanded states of the melanophores of the freshwater minnows, *Nocomis biguttatus* and *Semotilus atromaculatus*, result over a well-illuminated white bottom when light from above is either excluded or relatively weak. Melanophore ad-

justments in a situation where more light reaches the fish from below than from above appear to be made according to the degree of contrast in the visual field in the same way, though not to the same extent, as over a comparable normally illuminated dark bottom.

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